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Thre role of Bach1 in ultraviolet-A mediated human heme oxygenase-1 gene regulation

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**THE ROLE OF BACH1 IN ULTRAVIOLET-A MEDIATED
HUMAN HEME OXYGENASE-1 GENE REGULATION**

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A thesis submitted for the degree of Doctor of Philosophy

UNIVERSITY OF BATH
DEPARTMENT OF PHARMACY AND PHARMACOLOGY
JUNE 2008

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To Papa, Mom and Kunal,

Abstract

Up-regulation of Heme oxygenase-1 (HO-1) by ultraviolet-A (UVA) (320 - 380 nm) irradiation of human skin, provides a crucial defence mechanism against cellular oxidative stress. A general model for transcriptional activation of the HO-1 gene involves NF-E2-Related Factor 2 (Nrf2) as a transcriptional activator and Bach-1 as a negative regulator, both of which are involved in binding at MARE (Maf Recognition Element) sites in the promoter of the HO-1 gene. The role of Nrf2 in up-regulation of HO-1 and other Phase 2 genes is well established, but the mechanism underlying HO-1 repression mediated by Bach1 is still poorly understood. Cellular localisation of this repressor protein appears to hold the key to HO-1 regulation. In this study we show that UVA irradiation of cultured human skin fibroblasts enhances accumulation of Bach1 mRNA and protein several-fold and that endogenous Bach1 protein accumulates in the nucleus after 8h and may re-occupy the MARE sites once the HO-1 gene has been activated. Over-expression of Bach1, along with its binding partner MafK, represses basal and UVA-mediated HO-1 protein expression and silencing of Bach1 gene by Bach1-specific siRNAs cause robust enhancement of constitutive levels of HO-1 protein. UVA radiation treatment of cells in which Bach1 has been silenced, results in further induction of the HO-1 protein. We have also shown that Bach1 protein is exported from the nucleus with a 12 h delay following UVA irradiation. The data is consistent with a release of free cellular heme from microsomal heme containing proteins following UVA irradiation that promotes Bach1 export via the Crm1/Exportin1 pathway. Higher levels of Bach1 protein were observed in the nucleus and a low level of Nrf2 protein in the cell cytoplasm 48 h following UVA irradiation. Further UVA irradiation of cells 48 h after pre-irradiation causes further induction and accumulation of Bach1 protein in the nucleus while Nrf2 protein appears to stay at low (basal) levels in the cytoplasm. Our data are consistent with the concept that lack of Nrf2 nuclear accumulation and higher Bach1 protein in the nucleus contributes to the development of refractoriness to re-induction of HO-1 m-RNA and protein by UVA.

ABBREVIATIONS

AP1	Activator protein-1
APS	Ammonium persulphate
ARE	Antioxidant response element
Bach1	BTB and CNC homology-1
Bach2	BTB and CNC homology-2
BR	Bilirubin
BSA	Bovine serum albumin
BTB	Broad complex, tramtrack, bric-a-brac
BTB/POZ	Broad complex, tramtrack, bric-a-brac/poxvirus, zinc finger
BV	Biliverdin
bZip	Basic leucine zipper
CdRE	Cadmium response element
cGMP	Cyclic guanosine monophosphate
CLS	Cytoplasmic localisation sequence
CNC	Cap'n'collar
CO	Carbon monoxide
CP	Cystine proline
D ₂ O	Deuterium oxide
DFO	Desferrioxamine
DMSO	Dimethyl sulphoxide
dsRNA	Double-stranded RNA
DTT	1,4-dithio-DL-threitol
EDTA	Ethylene-diaminetetraacetic acid
EGTA	Ethylene-glycoltetraacetic acid
EMEM	Earle's modified essential medium
ER	Endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinase-1/2
GFP	Green fluorescent protein
GST	Glutathione S-transferase

H₂O₂ Hydrogen peroxide
Hb Hemoglobin
HO-1 Heme Oxygenase-1
HO-2 Heme Oxygenase-2
HO-3 Heme Oxygenase-3
HOIL-1 heme-oxidized IRP2 ubiquitin ligase-1
HRM Heme regulatory motifs
HSF-1 Heat shock factor-1
ICAM-1 Intercellular adhesion molecule-1
ICC Immunocytochemistry
IHABP Intracellular hyaluronic acid binding protein
IL Interleukines
INF γ Interferons γ
IRI Ischemia-reperfusion injury
IRP-2 Iron regulatory protein 2
Keap1 Kelch-like ECH-associated protein 1
LCR Locus control regions
LMB Leptomycin B
LPS Lipopolysaccharide
MARE Maf associated recognition elements
MAPK mitogen-activated protein kinase
NES Nuclear export signal
NF- κ B Nuclear factor- κ B
NO Nitric oxide
NQO1 NAD(P)H: quinine oxidoreductase
Nrf2 NF-E2-Related Factor 2
ORF Open reading frame
PAGE Polyacrylamide gel electrophoresis
PBS Phosphate buffered saline
PDGF Platelet-derived growth factor
PDT Photodynamic therapy

PFA Para-formaldehyde
PI Propidium iodide
PMSF Phenylmethylsulfonyl fluoride
PS Phosphatidylserine
REs Response elements
RNAi RNA inhibition
ROS Reactive oxygen species
SA Succinyl acetone (4,6-dioxoheptanoic acid)
SDS Sodium dodecyl sulphate
siRNA Small interfering RNA
StRE Stress response elements
TAE Tris-acetate buffer
TEMED N,N,N',N'-tetramethylethylenediamine
TGF Transforming growth factor
TNF- α Tumour necrosis factor- α
USF Upstream stimulatory factor
UVR Ultraviolet radiation
UVA Ultraviolet-A (320 – 380 nm)
UVB Ultraviolet-B (280 – 320 nm)
UVC Ultraviolet-C (200 – 280 nm)

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1. Introduction

1.1 Ultraviolet Radiation

1.1.1 Brief History of UV

The beneficial and adverse effects of sunlight to humans have been known since early civilisations. This knowledge about sunlight was based on religious and cultural beliefs. The understanding and knowledge of sunlight was mainly based on personal observation and was transferred from generation to generation. Scientific discovery and increase in knowledge concerning UV radiation in the solar range was a very long and gradual process. In 1865, Maxwell proposed a theory that light and sound are generated by the interaction of electric and magnetic fields and called them "electromagnetic waves". Maxwell's theory was confirmed by Hertz in 1882 by developing a method to measuring microwaves, which was the first empirical evidence for radiation beyond the UV-visible-infrared spectrum.

In 1900, Planck suggested that radiation is consisted of energy packets of "quanta" which were directly proportional to the frequency. He theorized that the energy (E) of "quanta" is equivalent to its frequency (ν, s^{-1}) and Planck's constant ($h = 6.625 \cdot 10^{-34}$)

$$E = h \cdot \nu$$

Later in 1905, Einstein's findings supported Planck's theory and he theorized that the "quanta" were massless particles of energy which are released by molecules and atoms upon light absorption. In 1928, Lewis called the Planck's "quanta", "photons". Bohr proposed that electrons absorb energy from light and emit it at wavelengths that correspond to the energy of the electrons. In 1926, a theory of wave mechanics was developed by Schrödinger, in which he considered electrons as waves rather than particles. These landmark theories provided a vital base for our current understanding of "light" as electromagnetic waves whose speed is proportional to wavelength and frequency. The spectrum of electromagnetic radiation has been divided into a number of

regions based on their frequencies. The region that falls into Ultraviolet (UVR) is the primary focus for this study.

1.1.2 Types of UV Radiation

In the last century, physicists developed a terminology for UV radiation based upon the physical properties. The region of UV below 180nm was called "vacuum UV" which is absorbed by air and can only be used in a vacuum. The terminology "near UV" was used to describe UV rays in the region between 290-400nm that reach the Earth's surface. The region between the near and vacuum UV regions, 180-290 nm was described as "far UV". Biologists used a different terminology that emphasized the effects of solar UV on living organisms. The term "UVA" was used for the region 320-400 nm that penetrated window glass and had physiological effects on organisms. The term "UVC" was used to refer to the solar region below 290 nm that was absorbed by the ozone layer. The term "UVB" was applied to the region between the UVC and UVA, i.e., 290-320 nm, and this region was believed to be responsible for the deleterious effects of sunlight on living organisms.

In photochemistry and photobiology the ultraviolet portion of the solar radiation has been divided into three wavelength ranges called UVA, UVB and UVC on the basis of differences in biological interactions. The short wavelength UVC region (190-290nm) is strongly absorbed by ozone and other molecules of the atmosphere. The UVC portion is not been able to reach earth's surface. Therefore, solar UV reaching the earth's surface contains UVB (290-320nm) and UVA (320-380nm) radiation. The total amount of UVR energy reaching the earth's surface is mainly composes of UVA (around 95%). In humans, the skin is the major organ exposed to UVR. In the skin, transmission of UV through skin increases with increase in wavelength. As described in the Figure 1, UVB penetrates mainly the epidermal layer while UVA radiation can penetrate not only the dermal but also the epidermal layer of human skin. UVA therefore effects more of the skin and subcutaneous tissue than UVB radiation (Tyrrell, 1995;Tyrrell, 1996a;Tyrrell,

1996b) . The main focus of this study will be the effects of UVA radiation on human skin cells.

Figure 1

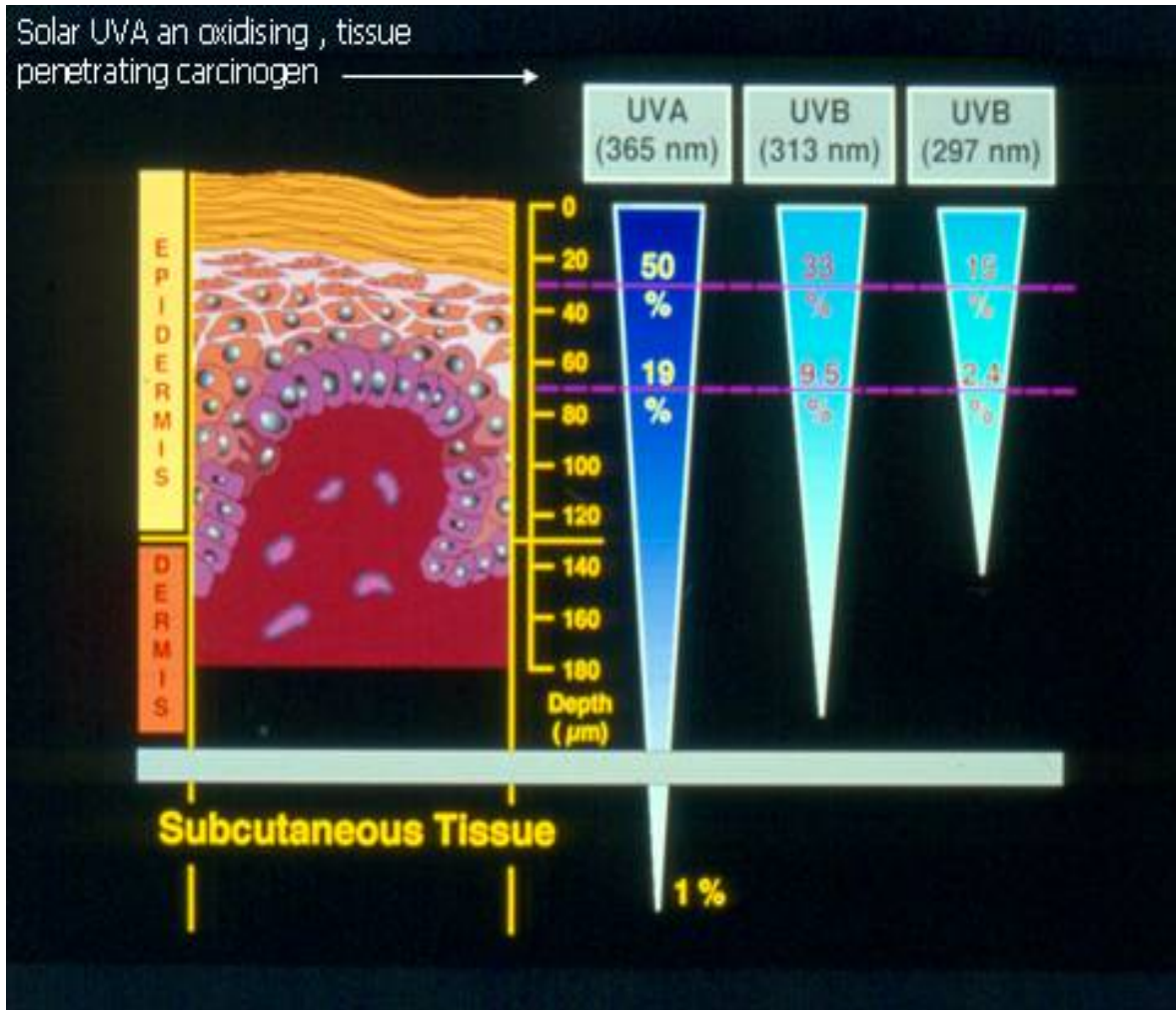


Figure 1

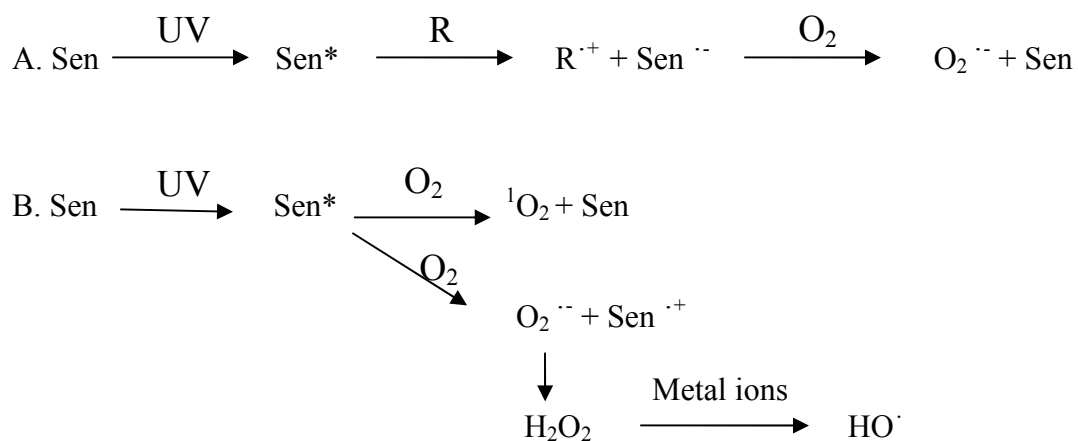
Penetration of UVA and UVB rays into the dermal and the epidermal layers of human skin

1.1.3 UVR-mediated photochemistry

The biological effects of UVR depend on their energy and the availability of the absorbing molecules. The cellular molecules (chromophores and photosensitizers) which absorb energy from UV photons include nucleic acids, amino acids (including tryptophan, tyrosine, phenylalanine, histidine and cysteine), NADH and NADPH, heme, quinones, flavins, porphyrins, carotenoids, 7-dehydrocholesterol, eumelanin and urocanic acid (UCA) (Tyrrell, 1973; Tyrrell et al., 1991; McCormick et al., 1976; Cunningham et al., 1985; Czochralska et al., 1984; McCormick et al., 1976). UVR mediated damage occurs mainly by two different mechanisms. The first is a direct mechanism by which UV photons donate energy to the UV absorbing molecules and cause damage to them. Upon absorbing a photon the cellular chromophores undergo changes in the electron distribution and normally create the reactive singlet state. In this state the excited molecules either loses energy by heat or emission of fluorescence or photoproducts will be generated via photoreactions. Alternatively and much more rarely the absorption of photon energy may lead to an excited triplet state which as for the singlet state can come back to the ground state by losing energy to photoreactions, photofluorescence or heat.

In the second mechanism the absorbing molecules work as a photosensitizer and generate reactive intermediates which are further involved in photoreactions and cause damage. Indirect photodamage via photosensitizers occurs through two pathways and depend on the properties of the photosensitizer. In the Type 1 mechanism the photosensitizer transfers one electron to the other cellular molecules through direct interaction and generates free radicals (figure 2). In this case, oxygen molecules are not required to produce reactive species. In the Type 2 mechanism the photosensitizers donate energy to oxygen molecules and generate highly reactive oxygen species commonly known as ROS. These ROS include a number of active metabolites including $^1\text{O}_2$, OH^\cdot , $\text{O}_2^{\cdot-}$ and peroxy radicals.

Figure 2



Sen=Sensitizers

Sen*=Sensitizers in excited state

R= Nucleic acids or aromatic amino acids

Figure 2 UV-mediated photo-reactions (A) In Type 1 mechanism the photosensitizers in excited state react with nucleic acids or aromatic amino acids (R) and generate free radicals. (B) The Type 2 reactions where the photosensitizers in excited state donate energy to oxygen molecules and generate highly reactive oxygen species.

1.1.4 UVA-mediated cellular stress

In humans, chronic and acute exposures to sunlight are associated with many pathological and physiological states. Acute exposure leads to erythema, sunburn, pigmentation, hyperplasia, immuno-suppression and vitamin D synthesis (de Laat and de Gruijl, 1996;Krutmann, 2001;de Gruijl, 2002;Tyrrell and Reeve, 2006) and the chronic exposure leads to skin cancer, cataract and skin ageing (Matsumura and Ananthaswamy, 2002;Agar *et al.*, 2004;Matsumura *et al.*, 2004;Halliday *et al.*, 2005). These pathological and physiological events are wavelength dependent and mainly occur as a result of cumulative solar UV dose.

At the cellular level it is well understood that UVA-mediated cell damage mainly occurs via ROS generated by photoreactions. Although UVA is absorbed weakly by cellular nucleic acids (Kielbassa *et al.*, 1997), UVA-mediated ROS can cause significant DNA damage and this includes strand breaks, DNA-protein crosslinks and cyclobutane-type pyrimidine dimers (Tyrrell, 1973). Singlet oxygen molecules generated via Type 2 reactions are highly reactive and very oxidative in nature. One of the earliest studies by Tyrrell and Pidoux suggested a role of singlet oxygen in UVA-induced cytotoxicity in human skin fibroblasts (Tyrrell and Pidoux, 1989). It was demonstrated that UVA treatment in the presence of a singlet oxygen lifetime enhancer (deuterium oxide, D₂O), increased UVA-mediated cytotoxicity while UVA treatment on the cells with singlet oxygen quenchers (histidine and sodium azide) reduced cell death by two-fold. This led to the conclusion that half of UVA-induced damage is singlet oxygen mediated (Tyrrell and Pidoux, 1989). Generation of superoxide anions by UVA mediated photoreactions causes photo-oxidation of NADH and NADPH (Cunningham *et al.*, 1985; Czochralska *et al.*, 1984) and also inactivates heme containing proteins (Garner *et al.*, 1995).

In some Type 2 reactions, hydrogen peroxide is also produced along with singlet oxygen. Initial *in vitro* studies by McCormick showed generation of hydrogen peroxide during UV-induced tryptophan degradation (McCormick *et al.*, 1976). Later, the same group showed generation of hydrogen peroxide following UVA exposure to cysteine

(McCormick et al., 1982). UVA interaction with NADH and NADPH (Cunningham et al., 1985; Czocharlska et al., 1984; McCormick et al., 1976) and macromolecules (Tyrrell et al., 1991) also generates hydrogen peroxide. Although hydrogen peroxide itself is reactive, far greater effects occur due to its role in the Fenton reaction. In this case cellular iron complexes react with the low level of hydrogen peroxide and generate a hydroxyl radical and a hydroxyl anion. Ferric iron is then reduced back to ferrous iron (Figure 3). Work has also shown that UVA is potentially capable of generating larger quantities of ROS because it leads to proteolytic degradation of the iron storage protein ferritin which leads to an increase in the free cellular iron contents (Pourzand et al., 1999).

Figure 3

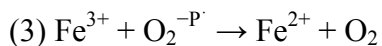
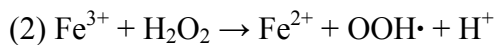
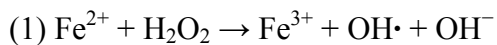


Figure 3 Iron-mediated generation of reactive oxygen species: Ferrous and Ferric irons react with hydrogen peroxide and generate highly reactive hydroxyl molecules.

1.1.5 UVA and gene activation

In human epidermal and dermal skin cells, UVA radiation leads to modulation of a wide array of genes that include transcription factors (Grether-Beck *et al.*, 1996b; Grether-Beck *et al.*, 1997b; Krutmann, 2000b), pro-inflammatory and anti-inflammatory cytokines (Chen *et al.*, 1995a; Baeuerle, 1998a), matrix metalloproteinases, heat shock proteins, mitogenic factors, cell surface adhesion molecules, genes involved in cutaneous malignancies, matrix proteins (Herrmann *et al.*, 1999), and heme oxygenase-1 (Keyse and Tyrrell, 1989b). UVA radiation also modulates the activity of the mitogen-activated protein kinase (MAPK) pathway (Basu-Modak and Tyrrell, 1993c; Klotz *et al.*, 1999a; Chen and Bowden, 1999a). UVA-mediated gene modulation mainly occurs via generation of singlet oxygen species and the recruitment of various signalling pathways (Basu-Modak and Tyrrell, 1993b).

UVA irradiation leads to activation of the transcription factor nuclear factor- κ B (NF- κ B) which regulates many genes involved in inflammatory and immune responses. UVA not only promotes nuclear accumulation of NF- κ B but it also increases DNA binding capacity (Vile *et al.*, 1995; Saliou *et al.*, 1999; Reelfs *et al.*, 2004). By modulating NF- κ B gene expression UVA also modulates expression of the NF- κ B target genes including IL1, IL6, IL8 and intercellular adhesion molecule (ICAM-1) in human cells (Chen *et al.*, 1995b; Baeuerle, 1998b). Recently work has shown that UVA irradiation promotes nuclear accumulation of NF-E2 related factor 2 (Nrf2) in mouse fibroblasts (Hirota A, 2005) and human skin fibroblasts (Edwards and Tyrrell unpublished observation). Nrf2 is a potent activator of all Phase 2 genes including Heme oxygenase-1 (HO-1), Glutathione S-transferase (GST) and NADPH quinone oxidoreductase (NQO1). UVA irradiation also activates expression of the activator protein-1 (AP-1) family of transcription factors which includes the Jun and Fos family of proteins (Bose *et al.*, 1999; Soriani *et al.*, 2000) which are involved in the regulation of complex mitogen-activated protein kinase (MAPK) signalling cascade of proteins (Basu-Modak and Tyrrell, 1993c; Klotz *et al.*, 1999b; Chen and Bowden, 1999b). UVA mediated activation of the AP2 family of proteins influences the process of morphogenesis and differentiation (Grether-Beck *et al.*,

1996a;Grether-Beck *et al.*, 1997a;Krutmann, 2000a). Proinflammatory cytokines induced by UVA include IL1alpha, IL6, TNF alpha and TGF beta 1. Anti-inflammatory cytokines IL10 are also induced (Tyrrell, 1996a;Tyrrell, 1996c).

1.1.6 The Antioxidant Defence Mechanism

Our body's defence mechanisms against oxidative stress can be divided into two basic pathways, constitutive and inducible. The constitutive pathways include antioxidant molecules, antioxidant enzymes and metal binding proteins. The antioxidant molecules such as carotenoids, glutathione, α -tocopherol, ascorbate and uric acid have very strong free radical scavenging properties. Enzymes like catalase, glutathione and superoxide dismutase also have antioxidant properties. The inducible pathways include Heme Oxygenase-1 (HO-1), Glutathione S-transferase (GST) and NADPH quinone oxidoreductase (NQO1). Among these inducible enzymes, HO-1 appears to be the most strongly induced. This study will mainly focus on the regulation of the inducible enzyme, HO-1.

1.2 Heme oxygenases

1.2.1 Types of Heme oxygenases

Heme oxygenase (HO) enzymatic activity was first discovered in the microsomal fraction of rat spleen, kidney and liver by Rudi Schmid and colleagues in the late 1960s' (Tenhunen, 1968;Tenhunen R, 1969). These studies characterised HO as a distinct enzyme that degrades heme into its metabolites and this was supported by work from Maines and Kappa demonstrating that heme degradation by HO is independent of Cytochrome P-450 mediated heme degradation (Maines and Kappas, 1974;Maines and Kappas, 1975). So far three isoforms of heme oxygenases have been identified, HO-1, HO-2 (Maines *et al.*, 1986;Trakshel *et al.*, 1986a) and HO-3 (McCoubrey, Jr. *et al.*, 1997a;Hayashi *et al.*, 2004). The HO-1 protein sequence has around 42% amino acid sequence homology with the HO-2 protein. In human cells, detailed *in situ* hybridization studies revealed that HO-1 and HO-2 are the products of two separate genes *hmox-1* and *hmox-2* respectively and they are localized on two separate chromosomes(*hmox-1*;22q12; *hmox-2*: 16p13.3) (Maines *et al.*, 1986;Cruse and Maines, 1988;Trakshel *et al.*, 1991;McCoubrey, Jr. *et al.*, 1992a). Both genes *hmox1*(~14kb) and *hmox2*(~12kb) have very similar structural organisation and include four introns and five exons(Muller *et al.*, 1987;Shibahara *et al.*, 1989;Alam *et al.*, 1994;McCoubrey, Jr. *et al.*, 1995). Several HO-1 and HO-2 cDNA clones have been reported (Shibahara *et al.*, 1985;Trakshel *et al.*, 1986b;Kageyama *et al.*, 1988b;Rotenberg and Maines, 1990;Rotenberg and Maines, 1991;Suzuki *et al.*, 1992;McCoubrey, Jr. *et al.*, 1992b). The third type of HO, HO-3 cDNA was cloned from rat tissues (McCoubrey, Jr. *et al.*, 1997b) and has 90% sequence similarity with HO-2.

HO-1 and HO-2 expression has been detected in most of the human tissue types tested so far. In humans, higher levels of HO-1 have been detected particularly in the spleen, liver and bone marrow compared with other tissues(Tenhunen, 1968;Tenhunen R, 1969). Higher HO activity has been detected in hematopoietic stem cells(Brown *et al.*, 1988;Abraham *et al.*, 1989;Abraham, 1991). However, in other cell and tissues types

constitutive levels of the HO-1 protein are very low but in the case of physical and chemical stimuli, there is rapid transcriptional activation of HO-1 (Section for details). The highest level of HO-2 expression have been observed in rat testis but it is also expressed at constitutive levels in many other tissues including kidney, liver, gut, brain and the nervous system (Maines *et al.*, 1986; Trakshel *et al.*, 1986c; Maines, 1997). The HO-2 gene is not activated in the response to environmental or physical stimuli. HO-3 is mainly expressed in rat brain, spleen, liver, thymus, kidney, testis, heart and prostate tissues (McCoubrey, Jr. *et al.*, 1997c; Hayashi *et al.*, 2004) but very little heme catabolic activity of HO-3 was observed when expressed in bacterial cells so it is possible that the previously reported HO-3 in rat may be a different transcript of HO-2.

Interestingly the HO-2 protein has two well characterized heme binding sites different from the heme catalytic domain (McCoubrey, Jr. *et al.*, 1997d). These additional heme binding sites carry the Cystein-Proline motif (CP motif), which are also known as heme regulatory motifs (HRM). The presence of the heme binding sites suggests that along with heme catabolism, HO-2 may have a role in heme sensing. It is believed that presence of the CP domains of HO-2 may provide additional binding sites for the heme molecule. The functional significance of these heme binding sites in HO-2 regulation is not clear but several reports propose that the heme binding sites may serve as a heme sensor (McCoubrey, Jr. *et al.*, 1997e; Ding *et al.*, 1999; Maines and Panahian, 2001).

1.2.2 HO enzymatic reaction

HO catalyzes heme (Fe-protoporphyrin-IX) by binding with heme in a 1:1 ratio and produced a single molecule of the open-chain α -isomer of biliverdin, biliverdin-IX α (Tenhunen R, 1969; Noguchi *et al.*, 1979). In this process and by using three moles of oxygen and reducing equivalents from NADPH:cytochrome P-450 reductase (Tenhunen, 1968; Tenhunen R, 1969; Yoshida *et al.*, 1980b; Noguchi *et al.*, 1983), HO cleaves Fe-protoporphyrin-IX at the α -methane position and release equimolar quantities of biliverdin-IX α , heme iron in the ferrous state and carbon monoxide (CO). Biliverdin-IX α

is further converted to bilirubin-IX α by NAD(P)H: biliverdin reductase. The graphical representation of the heme breakdown reaction is shown in the Figure 4.

The heme degradation reaction starts by the binding of heme with HO in a 1:1 ratio (Yoshida and Kikuchi, 1979). Detailed observations of the crystal structure of HO-1 revealed the existence of a heme pocket surrounded by a flexible bihelical structure (Schuller *et al.*, 1999a;Lad *et al.*, 2003b). The heme molecule in the HO pocket contacts two glycine residues situated at Gly-139 and Gly-143 in the distal helix domain (Schuller *et al.*, 1999b).The histidine molecule at the His-25 position in the HO-1 protein structure works as a heme iron ligand. The HO-bound heme works as a substrate and a cofactor in order to trigger the reaction. The NADPH-dependent reduction of heme starts in the heme-HO complex by the binding of heme iron with an oxygen molecule and forms an intermediate oxyferrous molecule which accepts an electron from the electron donor which is NADPH (Tenhunen R, 1969;Yoshida and Kikuchi, 1974b;Yoshida and Kikuchi, 1978b;Yoshida *et al.*, 1980a;Yoshinaga *et al.*, 1982) and converts the oxyferrous intermediate molecule to the peroxy intermediate, ferric hydroperoxide. This ferric hydroperoxide hydroxylates the heme ring at the α -methene bridge carbon and release α -meso-hydroxy heme (Wilks and Ortiz de Montellano, 1993;Yoshida and Migita, 2000;Lad *et al.*, 2003a). Following two more oxidation steps the carbon molecule from the α -methane bridge releases a CO molecule, verdoheme which is converted to ferribiliverdin- IX α (Yoshida and Kikuchi, 1974a;Yoshida and Kikuchi, 1974c;Yoshida *et al.*, 1982a;Yoshida *et al.*, 1982b;Kikuchi and Yoshida, 1983;Migita *et al.*, 1998;Sakamoto *et al.*, 1999). After one more reduction step ferrous iron is released from biliverdin complex (Yoshida and Kikuchi, 1978a). Both HO-1 and HO-2 follow the identical reactions to degrade heme. It is noteworthy that rat HO-1 and HO-2 have important differences in their biochemical properties. Thus differences in thermostability, substrate Km values and immunoreactivity between the two enzymes have been reported (Maines *et al.*, 1986;Trakshel *et al.*, 1986d).

Figure 4

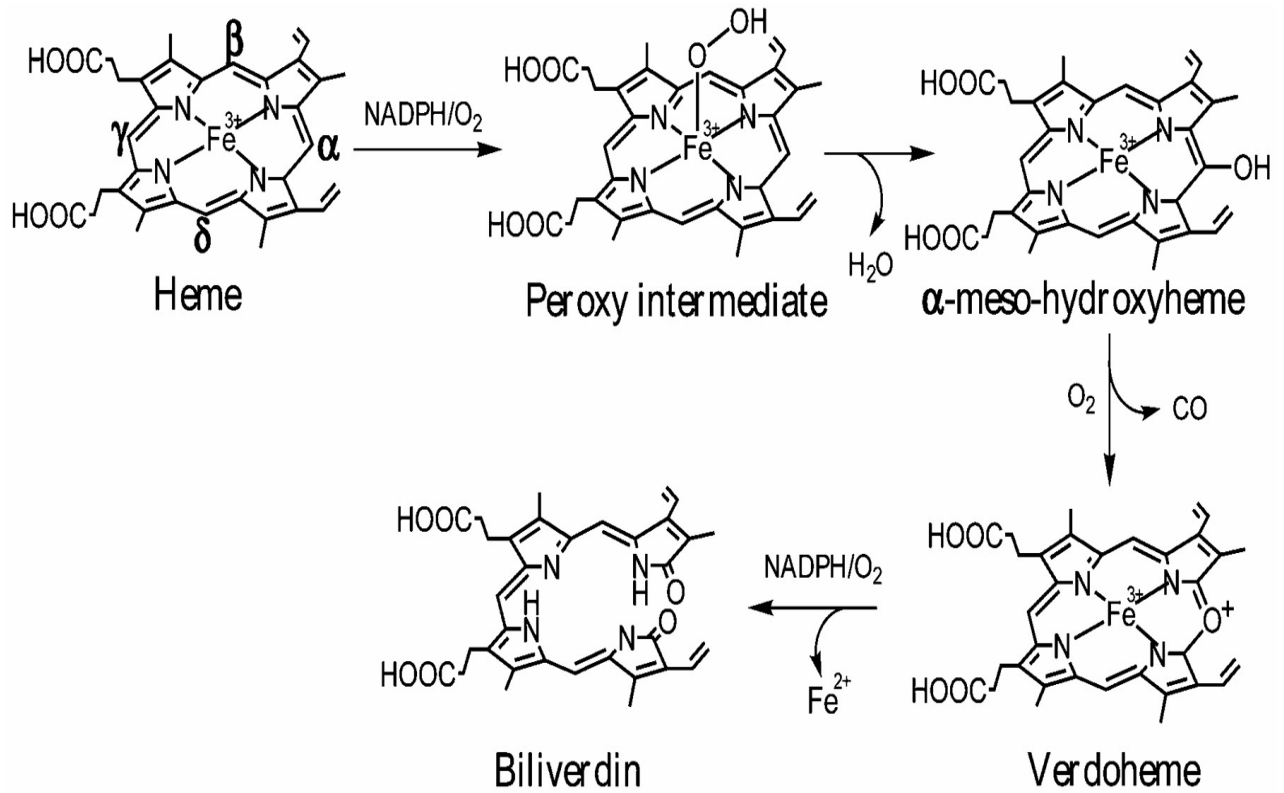


Figure 4

Heme oxygenase-mediated heme catabolism reaction where heme oxygenases bind with heme molecule and generate biliverdin, iron and carbon monoxide. (Figure adapted from Lad et al., 2003)

1.2.3 Sub-cellular distribution of HO

HO-1 is a well characterised protein associated with the endoplasmic reticulum (ER). Both HO-1 and HO-2 have a hydrophobic region in their C-terminal regions which help them to anchor in the cellular membrane (Shibahara *et al.*, 1985; McCoubrey, Jr. and Maines, 1993). Several recent studies reported HO-1 localisation in mitochondria, caveolae and the cell nucleus. Dennery and colleagues detected HO-1 protein signals in the cell nucleus following hypoxia, heme or heme/hemopexin treatments (Lin *et al.*, 2007). The study showed that under stress conditions the C-terminal region of HO-1 protein is cleaved and the N-terminal region of HO-1 protein migrates into the cell nucleus. This nuclear localised HO-1 protein lacks 52 amino acids from the C-terminal region and its molecular weight is around 27kD compared to the intact HO-1 protein (32kD). The nuclear migration of the HO-1 protein resulted in loss of HO enzymatic activity. It was proposed that the nuclear migration of HO-1 may play a crucial role in modulation of several transcription factors including AP-1 protein. Interestingly overexpression of HO-1 resulted in an increase in its own promoter activity; therefore it was proposed that the HO-1 protein may play a role in its own transcriptional regulation (Lin *et al.*, 2007).

HO-1 signals have also been detected in the mitochondria. The protein migrates from the ER to the cytochrome c-containing fraction following heme and lipopolysaccharide (LPS) treatments in mouse endothelial cells (Kim *et al.*, 2004b). HO-1 is also detected in the plasma membrane caveolae and may associate with the structural protein of caveolae, caveolae-1. Along with HO-1, biliverdin reductase, HO-2 and NADPH: cytochrome P-450 reductase also has been detected in the caveolae. Interestingly, silencing of caveolin-1 by the antisense approach increases HO enzymatic activity and overexpression of caveolae-1 resulted in a decrease in LPS-mediated HO-1 induction. Therefore it has been proposed that caveolin-1 plays a role in the HO-1 signalling mechanism via small gaseous second messengers which originate in the caveolae (Kim *et al.*, 2004a).

1.2.4 UVA mediated HO-1 gene activation

Induction of HO-1 appears to be a general response to oxidative stress in biological systems. In the late 1980's several reports suggested induction of a 32kD protein following various oxidative stress agents (Maines and Sinclair, 1977b; Hiwasa *et al.*, 1982a; Taketani *et al.*, 1988a; Keyse and Tyrrell, 1989a). In 1989, a landmark report from our laboratory identified HO-1 as the 32kD protein which expresses highly following UVA and hydrogen peroxide treatments in human skin fibroblasts (Keyse and Tyrrell, 1989d; Keyse and Tyrrell, 1990). Both UVA and hydrogen peroxide release heme, the substrate of the HO-1 reaction, from microsomal heme-containing proteins and this correlates with UVA-mediated HO-1 activation (Kvam *et al.*, 1999). Further to this, Basu-Modak and Tyrrell have shown that UVA-mediated generation of singlet oxygen species plays a central role in activation of the *hmx-1* gene. In this report it has been shown that UVA irradiation in presence of D₂O, which enhances the half-life of singlet oxygen, further increases accumulation of HO-1 mRNA, while UVA irradiation in presence of the singlet oxygen quenchers, sodium azide or histidine decreases HO-1 expression (Basu-Modak and Tyrrell, 1993a). In addition, UVA treatment with the natural singlet oxygen quencher, beta carotene, suppresses UVA mediated HO-1 activation in human skin fibroblasts (Trekli *et al.*, 2003). Taken together, these observations are consistent with the concept that both the substrate heme and UVA mediated release of singlet oxygen play a major role in UVA mediated HO-1 activation. Interestingly, total refractoriness to re-induction of the *hmx-1* gene was observed when a challenge dose of UVA was given 48h following the first dose (Noel and Tyrrell, 1997).

1.2.5 HO-1 inducers

In the last two decades HO-1 induction has been established as a ubiquitous cellular response to combat cellular stress in many experimental models. HO-1 is activated by many ROS generating systems including UVA (Keyse and Tyrrell, 1987;Keyse and Tyrrell, 1989f), hydrogen peroxide (Keyse and Tyrrell, 1987;Keyse and Tyrrell, 1989e), menadione (Keyse and Tyrrell, 1989g), heavy metals like cadmium and cobalt chloride (Maines and Sinclair, 1977a;Hiwasa *et al.*, 1982b;Taketani *et al.*, 1988b;Keyse and Tyrrell, 1989h), metalloporphyrins including heme (Alam *et al.*, 1989;Takeda *et al.*, 2000;Alam *et al.*, 2003), cobalt and zinc Metalloporphyrins (Mitani *et al.*, 1993), thiol reactive substances such as sodium arsenite(Kageyama *et al.*, 1988a;Keyse and Tyrrell, 1989c;Applegate *et al.*, 1991b), iodoacetamide (Caltabiano *et al.*, 1986), diethylmaleate (Shelton *et al.*, 1986), nitric oxide derivatives including NO gas (Marquis and Demple, 1998), NO donors (Motterlini *et al.*, 1996;Durante *et al.*, 1997;Hartsfield *et al.*, 1997;Chen and Maines, 2000), peroxyxynitrite (Foresti *et al.*, 1999;Hara *et al.*, 1999b), inflammatory cytokines such as IL1 α , β (Terry *et al.*, 1998a;Terry *et al.*, 1999a), IL6 ,IL11 (Fukuda and Sassa, 1993), tumour necrosis factor- α (TNF α) (Terry *et al.*, 1998b;Terry *et al.*, 1999b), transforming growth factor β (TGF β) (Ning *et al.*, 2002), interferon γ (INF γ) (Udono-Fujimori *et al.*, 2004) platelet-derived growth factor (PDGF) (Kutty *et al.*, 1994;Durante *et al.*, 1999), lipopolysaccharide (LPS) (Lutton *et al.*, 1992;Camhi *et al.*, 1995b), lipid metabolites (Koizumi *et al.*, 1992;Kronke *et al.*, 2003b;Liu *et al.*, 2004), tumour promoters eg. tissue plasminogen activator (TPA) (Hiwasa *et al.*, 1982c;Hiwasa *et al.*, 1983;Hiwasa and Sakiyama, 1986;Alam and Den, 1992b;Muraosa and Shibahara, 1993b), hyperoxia (Takahashi *et al.*, 1998;Fogg *et al.*, 1999a;Lee *et al.*, 2000a), hypoxia(Murphy *et al.*, 1991), curcumin (Motterlini *et al.*, 2000;Balogun *et al.*, 2003b) and exercise (Thompson *et al.*, 2005).

1.2.6 Functional significance

Induction of HO-1 has been shown to be crucial for protection against cell damage (Otterbein and Choi, 2000; Poss and Tonegawa, 1997; Ryter and Tyrrell, 2000). The beneficial effects of HO and its by-products have been well established in various experimental models (See section 1.2.7 for more details). A role for HO-1 has been proposed in many disease conditions including psoriasis (Detmar *et al.*, 1994), chronic obstructive lung disease (Carter *et al.*, 2004; Xia *et al.*, 2006) atherosclerosis (Ishikawa *et al.*, 1997; Morita, 2005; Siow *et al.*, 1999; Wang *et al.*, 1998), transplant rejection (Bach, 2006; Hancock *et al.*, 1998; Soares *et al.*, 1998; Woo *et al.*, 2000), Alzheimer's disease (Calabrese *et al.*, 2004; Ishizuka *et al.*, 2002; Smith *et al.*, 1994), acute renal injury (Akagi *et al.*, 2005; Goncalves *et al.*, 2006), hypertension (Ishizaka *et al.*, 1997; Vera *et al.*, 2006) toxic nephropathy (Agarwal *et al.*, 1995; Horikawa *et al.*, 1998), endotoxic shock (Lee and Chau, 2002) and gastrointestinal diseases (Guo *et al.*, 2000).

Functional significance of HO-1 was further confirmed by development of the *hmx-1* deficient mouse strain. The HO-1^{-/-} animals were more sensitive to oxidant stimuli such as heme, cadmium, hydrogen peroxide and paraquat (Poss and Tonegawa, 1997a; Poss and Tonegawa, 1997b). Exposure of several stress agents to HO-1 deficient mice resulted in chronic liver injuries (Poss and Tonegawa, 1997c), acute renal injury, significant decrease in renal function, tubular injury (Nath *et al.*, 2000b), enlargement of right ventricular (Yet *et al.*, 1999), increased apoptosis, necrosis and mortality (Poss and Tonegawa, 1997d; Nath *et al.*, 2000a). The HO-1 deficient mice showed increased deposition of iron in renal cortical tubules, many signs of a progressive chronic inflammation characterized by hepatosplenomegaly, lymphadenopathy, leukocytosis, hepatic periportal inflammation and occasionally glomerulonephritis (Poss and Tonegawa, 1997e). The importance of HO-1 in humans was confirmed by the discovery of a severely sick child who was characterized as having a HO-1 deficiency. Similar to the HO-1 knock out mice, the HO-1 deficient patient suffered from anaemia, persistent proteinuria, hematuria, coagulation defects, hyperlipidemia, stunted growth, iron deposition, leukocytosis, increases levels of heptoglobin, heme, ferritin and lower levels

of serum albumin. Microscopic examination of the patient's renal biopsy showed lymphocyte infiltration, detachment of the glomerular capillary endothelium and mesangial cell proliferation (Yachie *et al.*, 1999).

1.2.7 Mechanism of HO-1 protection

Cellular protection on a consequence of HO activity involves several pathways. It is commonly known that almost all of the HO-1 inducers cause change in the cellular redox balance. It has been proposed that many oxidative stress conditions promote a cellular pro-oxidant state by production of various reactive oxygen species or by decreased cellular reductants (Applegate *et al.*, 1991a). HO-1 induction appears to be general response to maintain the cellular homeostasis by driving back the cellular redox status towards the anti-oxidant state. As described above, the HO reactions degrade free cellular heme to iron, CO and biliverdin 9 alpha which will eventually be converted to bilirubin by biliverdin reductase. The substrate of the HO-1 reaction, heme itself, has well-characterised pro-inflammatory roles and can produce ROS via the Fenton reaction. Accumulation of heme in the cellular environment can cause severe damage to the many cell types tested (Balla *et al.*, 1991; Balla *et al.*, 1993) and removal of excess heme prevents potential cellular injuries. In addition to free heme degradation by the HO reaction, all the by-products may contribute to cellular protection by various mechanisms.

As mentioned in section 1.2.2, during the HO-mediated heme degradation reaction, HO catalyses the alpha meso bridge of the heme molecule and releases endogenous CO molecules in an equimolar quantity. CO has been well characterised as a poisonous gas. CO binds to Hb with very high affinity compare to O₂ and depletes the supply of O₂ to body organs. Adverse effects of CO have been well characterised in various disease conditions including asthma, diabetes and bronchitis. Until recently, CO was recognised as an unwanted hazardous by-product of the HO reaction. Interestingly many scientists have observed significant improvements in transplant organ survival in rats when they have been supplied with excess CO (Neto *et al.*, 2004). It has been reported that CO

exerts vasodilatory effects via cGMP dependant smooth muscle relaxation. CO activates the cGMP by binding with the heme molecule of the soluble guanylyl cyclase (sGC) (Furlong *et al.*, 1987; Ramos *et al.*, 1989; Utz and Ullrich, 1991; Kubes *et al.*, 1991; Cooke and Tsao, 1993; Yu *et al.*, 1994; Linas *et al.*, 1997; Kajimura *et al.*, 2002). Several other studies suggested cGMP-mediated effects of CO in neurotransmission (Verma *et al.*, 1993; Snyder *et al.*, 1998), protection of pancreatic β cells from apoptosis (Gunther *et al.*, 2002), inhibition of platelet aggregation (Brune and Ullrich, 1987), bronchodilation (Cardell *et al.*, 1998; Fujita *et al.*, 2001) and vascular smooth muscle proliferation (Morita and Kourembanas, 1995b).

An antiapoptotic role of CO was first observed in the mouse fibroblasts and endothelial cells via inhibition of TNF- α mediated apoptosis (Brouard *et al.*, 2000; Petrache *et al.*, 2000). Later on the same effect was observed by overexpression of HO1 protein in *in vitro* models. CO is also directly involved in induction of NF- κ B-dependent antiapoptotic proteins to protect against TNF- α mediated apoptosis (Brouard *et al.*, 2002). An antiapoptotic role of CO in various tissue injuries (mainly ischemia perfusion) and organ transplant has been well studied in various *in vitro* and *in vivo* models. Further to this, various studies have demonstrated the role of endogenous (via HO reaction) and exogenous CO in anti-inflammatory effects in various *in vitro* and *in vivo* models. CO has been implicated in modulation of various inflammatory pathways by modulating production of pro-inflammatory cytokines (TNF- α , IL1 β , IL2, IL6 and microphage inflammatory protein- β (MIP β)). It has also been shown that by interaction with MAPK pathways CO increases expression of the anti-inflammatory cytokine IL10 (Ryder *et al.*, 2006). For example CO has been shown to decrease T cell proliferation and IL2 production via inhibition of the extracellular signal-regulated kinase (ERK) pathway (Otterbein *et al.*, 1999). In several disease models, an antiinflammatory role of CO has been demonstrated. For example, CO inhibits IL6 production via the JNK pathway in sepsis following either exogenous CO administration or over-expression of HO-1/ induction of HO-1 by known inducers (Morse *et al.*, 2003). CO's role in anti-proliferative mechanisms has also been well studied. CO inhibits cell growth by influencing expression of cell cycle related factors (Morita and Kourembanas, 1995a).

The bile salts, bilirubin and biliverdin are the other by-products of the HO enzymatic reaction. In 1976, it was shown that bilirubin effectively quenches singlet oxygen molecules in organic solvent and prevents photo-oxidation of hydrocarbons (Stevens and Small, Jr., 1976). Later, Stocker and his group showed bilirubin as the strongest scavenger of peroxy radicals in the serum and demonstrated that both bilirubin and biliverdin exert strong antioxidant properties and provide cytoprotection against oxidative injuries (Stocker and Ames, 1987; Stocker *et al.*, 1987a; Stocker *et al.*, 1987b; Stocker and Peterhans, 1989; Neuzil and Stocker, 1993; Neuzil and Stocker, 1994). Subsequent studies revealed that both exogenous administration and HO-1-mediated BV and BR generation provide crucial cell protection. Along with potent antioxidant properties, BR also exerts anti-inflammatory effects (Hayashi *et al.*, 1999a). Increased HO-1 expression inhibited leukocyte adhesion. Interestingly the same effects were observed by exogenous supplementation of BR and BV but not CO (Hayashi *et al.*, 1999b). Increased concentrations of serum BR levels have been shown to decrease risk of atherosclerosis, coronary artery disease (Mayer, 2000; Schipper, 2004), cancer mortality (Temme *et al.*, 2001), resolution of asthma symptoms (Ohrui *et al.*, 2003), and a decreased incidence of the retinopathy associated with prematurity (Heyman *et al.*, 1989). Exogenous supplementation of BR has been shown to provide crucial protection against the ischemia-reperfusion injury (IRI) associated with organ transplantation (Clark *et al.*, 2000; Adin *et al.*, 2005). As for BR, exogenous supply of BV has been shown to have several beneficial effects in different experimental models. For example, BV supplementation increases survival of heart allografts (Yamashita *et al.*, 2004), increases expression of antiapoptotic genes (Fondevila *et al.*, 2004b) and decreases apoptosis, pro-inflammatory cytokines and leukocyte infiltration (Fondevila *et al.*, 2004a).

There is no apparent cytoprotective role of free iron in cells. On the contrary, free iron is highly pro-oxidant and generates hydroxyl radicals and lipid-derived peroxy radicals which cause cellular damage (Aust *et al.*, 1985; Halliwell and Gutteridge, 1992). It has been shown that induction of HO-1 leads to an increase in the iron-storage protein, ferritin (Vile and Tyrrell, 1993). Ferritin reduces the free iron in the cellular environment and provides defence against oxidative stress that has the potential to be exacerbated by

iron. It has been shown that overexpression of ferritin provides cytoprotection against oxidative injury (Balla *et al.*, 1992;Cermak *et al.*, 1993;Lin and Girotti, 1997).

1.2.8 HO-1 gene promoter and regulation

The physiological importance of HO-1 has increased of interest in understanding the transcriptional regulation of the HO-1 gene. Numerous studies of the HO-1 promoter have shown an abundance of consensus binding sites for several transcription factors including $\text{Nf-}\kappa\text{B}$, Nrf2, Bach1, AP1, AP2, Sp1 and functionally identified response elements (REs) or cis-acting DNA sequence elements. Initial studies suggested the presence of multiple REs in the proximal and distal promoter regions of the mouse and human HO-1 gene. In the human HO-1 gene, the response elements (REs) and binding sites have been observed up to -15kb upstream region of the transcriptional start site. The abundance of multiple REs and transcription factor binding sites suggest a potential role of these factors in HO-1 gene regulation. These binding sites may play an active role under different conditions and in different cell types consistent with the given the structural and functional diversity observed between the HO-1 inducers. It is noteworthy that there is considerable inter-species and intra-species variation in the positions and functional role of these binding sites in HO-1 regulation.

It has been shown in a rodent model that the HO-1 gene is regulated by two upstream enhancer regions residing at -4kb and around -10kb of the mouse HO-1 gene core promoter, called E1 and E2 respectively. (Alam, 1994; Alam *et al.*, 1994; Alam *et al.*, 1995; Alam and Cook, 2003) These enhancer regions are activated by many stress-inducing agents including cadmium, arsenite, heme, hydrogen peroxide, TPA and other heavy metals (Camhi *et al.*, 1995a; Inamdar *et al.*, 1996). Both these enhancer regions contain three stress response elements (StRE) (Inamdar *et al.*, 1996) which are also the recognition sequence for the small Maf family of proteins and are therefore also known as MARE (Maf Recognition Elements) sites. The StRE is structurally and functionally similar to the MARE and the antioxidant response element (ARE) as well as the heme responsive element (GCNNNGTCA) consensus and an intrinsic TPA responsive element (TRE)/ AP-1 site (Alam and Den, 1992a; Inamdar *et al.*, 1996). These elements will be collectively referred to as the MARE for the purposes of this thesis. The MARE site is a 10 base pair core sequence (T/G)GCTGAGTCAC, which also carries potential DNA

binding sites for the AP1 family of proteins (T/G) GAGTCA. Detailed promoter studies of the human HO-1 promoter reveal that the human HO-1 promoter also carries two enhancer regions at the -4 kb and -9 kb upstream regions which are called region A and B respectively (Takeda *et al.*, 1994a; Hill-Kapturczak *et al.*, 2001; Kataoka *et al.*, 2001b; Kitamuro *et al.*, 2003a; Chen *et al.*, 2003b; Hill-Kapturczak *et al.*, 2003c; Hill-Kapturczak *et al.*, 2003e). Here it is important to mention that unlike the E1 and E2 regions of mouse which both contain three MARE, human A region contains one cadmium response element (CdRE) in the middle of the two MARE sites at the -4kb region while the B region contains three MARE sequences. Several reports have suggested that these regions, A and B, are essential for induction of the human HO-1 gene in response to heme, cadmium (Takeda *et al.*, 1994a; Hill-Kapturczak *et al.*, 2003b), NO donor compounds (Hara *et al.*, 1999a), oxidized phospholipids (Favatier and Polla, 2001b; Kronke *et al.*, 2003a), cigarette smoke (Favatier and Polla, 2001a) gold compounds (Kataoka *et al.*, 2001a) and shear stress (Chen *et al.*, 2003a).

A bioinformatics database search (Transfac version 6.0) suggested the existence of MARE sites at -4 and -9.1 kb upstream of the human ho1 gene (Raval and Tyrrell, unpublished observations). Interestingly we also observed the existence of various partial or half-MARE sites near both the enhancer regions and also distributed over a wide range of 5' region up to -10kb in the human HO-1 promoter. The full role of these distal partial MAREs and their role in HO-1 gene regulation has yet to be understood, but recent evidence suggests the possible involvement and co-operativity of these distal MARE sites in HO-1 gene activation (Yoshida *et al.*, 1999; Lu *et al.*, 2000; Lee *et al.*, 2000b). A recent report has indicated that a region between -9.1/-11.6kb is important in the response to organic hydroperoxides (Hill-Kapturczak *et al.*, 2003d). In the human HO-1 gene, Agarawal and colleagues demonstrated that along with enhancer regions A and B, the exons also play a crucial role in achieving full HO-1 induction in response to heme and cadmium (Hill-Kapturczak *et al.*, 2003d).

The MAREs represent targets of multiple dimeric proteins generated by intra-family homodimerization or intra- and inter-family heterodimerization of individual members of

the Jun, Fos, CREB, ATF, Maf, and the Cap'n'collar/ basic-leucine zipper (CNC-bZIP) subclasses of the basic leucine zipper (b-ZIP) superfamily of transcription factors. Members of each of these families have been implicated in *ho-1* gene regulation (Numazawa *et al.*, 1997; Oguro *et al.*, 1998; Elbirt *et al.*, 1998; Alam *et al.*, 2000c; Kietzmann *et al.*, 2003; Kierner *et al.*, 2003; Alam and Cook, 2003; Shibahara, 2003; Balogun *et al.*, 2003a; Kronke *et al.*, 2003c). A general model for HO-1 regulation involves the Maf regulatory network in which small Maf proteins play a central role (Figure 5). At MARE sites the repressor protein Bach1 competes with the activator protein Nrf2 to bind with the small Maf proteins. Under normal non-stimulated conditions Bach1 occupies the MARE sites of the HO-1 promoter, thereby represses the HO-1 gene and maintains the constitutively low level of HO-1 protein expression (Sun *et al.*, 2002; Sun *et al.*, 2004b). *In vivo* experiments on Bach1 deficient mice have shown constitutively high expression of HO-1 in many tissue types (Sun *et al.*, 2002). In normal stress-free conditions, Nrf2 activity is suppressed by the cytoplasmic protein Keap1 which interacts with the Nrf2 in cytoplasm and works as an anchor protein (figure 5A) (Itoh *et al.*, 1999a). When cells are exposed to oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus (figure 5B). In the case of extracellular heme treatment, heme decreases the DNA binding capacity of the MARE bound Bach1 and releases Bach1 from MARE sites (Ogawa *et al.*, 2001f). Heme also stabilises the short-lived Nrf2 protein in the nucleus which then occupies the MARE sites of the HO-1 promoter and activates the HO-1 gene (Itoh *et al.*, 1999a). A major focus of this study will be the role of Bach1 in regulation of HO-1.

Figure 5

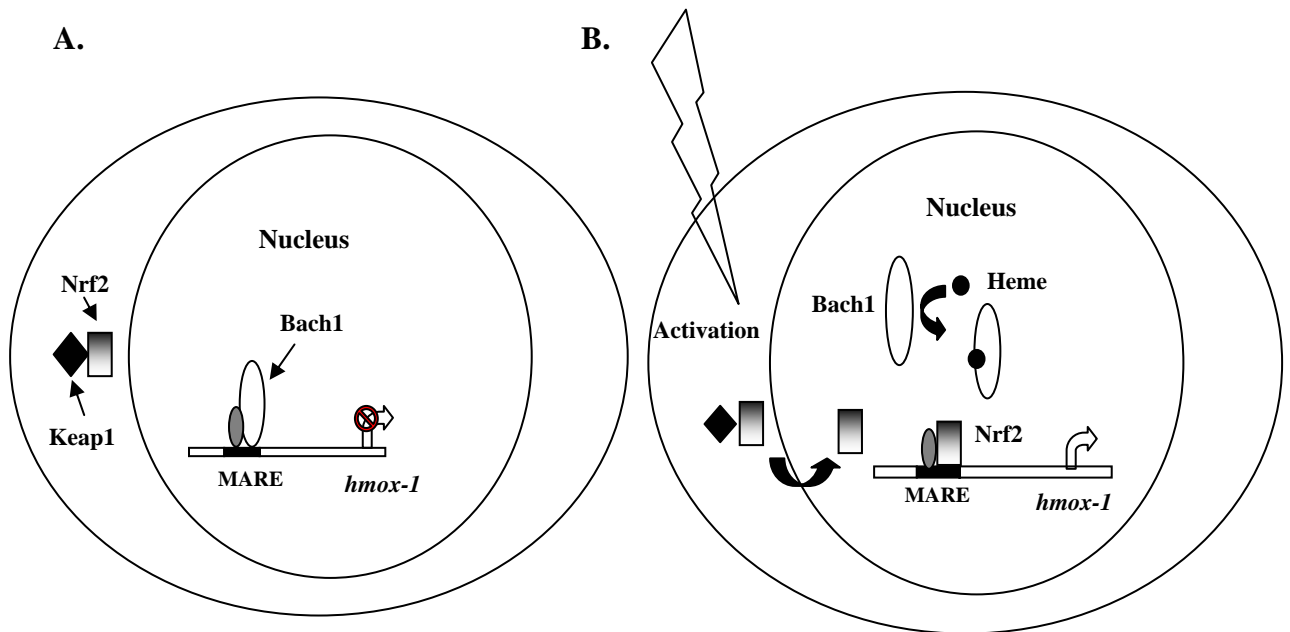


Figure 5: HO-1 gene regulation

A. In normal conditions, Bach1 protein stays in the cell nucleus and occupies the MARE sites of the HO-1 promoter in order to keep the HO-1 gene suppressed, while Nrf2 activity is inhibited by Keap1 binding in the cytoplasm. **B.** Under stress conditions, heme binds to the MARE-bound Bach1 protein and releases Bach1 from MARE sites. At the same time, Nrf2 protein is accumulated in the nucleus which occupies the MARE site to activate the HO-1 gene.

1.2.9 Proximal Promoter

In the human *ho-1* gene, a number of proximal promoter elements have been identified in the area immediately adjacent to the transcriptional start site (Shibahara *et al.*, 1989; Tyrrell *et al.*, 1993; Sikorski *et al.*, 2004), which include binding sites for heat shock factor-1 (HSF-1) (Shibahara *et al.*, 1989), activator protein-2 (AP-2) (Lavrovsky *et al.*, 1993; Lavrovsky *et al.*, 1994), nuclear factor-kappa B (NF- κ B) (Lavrovsky *et al.*, 1993; Lavrovsky *et al.*, 1994), the upstream stimulatory factor (USF) (-51/-42) STAT-3 (Deramaudt *et al.*, 1999), basic helix loop helix (bHLH) proteins (-156/-147) (Nascimento *et al.*, 1993; Muraosa and Shibahara, 1993a) and ETS binding sites (EBS) (Deramaudt *et al.*, 2001). The other important region of the human *ho-1* gene promoter is in the region between -1976/-1655 with abundance of REs which include consensus binding sites for AP-1 (-1872), STATx (-1751), c-Rel (-1723), hepatocyte nuclear factor-1 (HNF-1) (-1709), HNF-4 (-1787), and GATA-X (-1803,-1672) (Takahashi *et al.*, 1997; Takahashi *et al.*, 2002). Here it is important to mention that these REs situated in between -1976 and -1655 bp region are functionally active in HepG2 cells while they are functionally inactive in HeLa cells (Takahashi *et al.*, 1997; Takahashi *et al.*, 2002).

An other important region has been identified in the proximal region between -198 kb and -258kb of the human *ho-1* promoter which carries a (GT)_n repeat (Yamada *et al.*, 2000a). A correlation between the number of repeats and severity of disease conditions has been proposed in patients with coronary artery disease (Kaneda *et al.*, 2002b; Chen *et al.*, 2002b), vascular restenosis after balloon angioplasty (Exner *et al.*, 2001b) and amphysema (Mazza *et al.*, 2003). It has been proposed that the individuals with shorter repeats, S allele (<25 repeats), demonstrate higher levels of HO-1 and are therefore less prone to the disease condition, whereas individuals with longer repeats, L allele (>25 repeats) have lower levels of HO-1 and therefore are more susceptibility to the disease (Yamada *et al.*, 2000b). However there have been many negative reports that argue against the direct involvement of number of GT repeats and disease conditions (Exner *et al.*, 2001a; He *et al.*, 2002; Kaneda *et al.*, 2002a; Chen *et al.*, 2002a). A study in this laboratory has suggested no significant link between the poly GT regions of the HO-1

promoter and the patients with psoriatic disease (Raval, Magaraggia and Tyrrell unpublished data). Deletion of the entire poly GT region resulted in higher levels of basal HO-1 promoter reporter activity (Yamada *et al.*, 2000c). The difference in the GT repeats has been assumed to play a crucial role in the formation of DNA structure and also has been proposed to be responsible for the alternative DNA structure known as zDNA (Naylor and Clark, 1990). However in the case of the *ho-1* gene, a link between formation of zDNA structure and its role in regulation of *ho-1* expression has not yet been made.

1.2.10 Differences between the mouse and human *ho-1* gene

Although the mouse and human *ho-1* genes have ~90% sequence homology there are significant differences in the promoters in the regulation of the two genes (Figure 6). In humans the presence of the (GT)_n repeat has been identified while this region is completely absent in the mouse gene (Yamada *et al.*, 2000d). Furthermore in the human HO-1 promoter, the enhancer region at -4kb (region A) contains a potential cadmium response element (CdRE) in between the two StREs (Takeda *et al.*, 1994b; Alam *et al.*, 2000b) while in the mouse gene the enhancer region E1 carries three StREs and a CdRE is located immediately downstream of the E1 region (Takeda *et al.*, 1994c; Alam *et al.*, 1999; Alam *et al.*, 2000a). Consistent with this, the mouse and human *ho-1* gene respond differently under various stress conditions. For example, the *ho-1* gene is induced in response to heat shock in rodent models and has been identified as a heat shock protein whereas the human *ho-1* gene is not induced by heat shock (Sato *et al.*, 1990; Okinaga *et al.*, 1996; Shibahara *et al.*, 2002b). The enhancer region (E1) in the mouse *ho-1* promoter regulates the *ho-1* gene in response to oxidized lipids, hyperoxia and hydrogen peroxide (Lee *et al.*, 1996; Choi and Alam, 1996; Li *et al.*, 2000) while the enhancer region A is not active under these conditions in the human *ho-1* gene (Fogg *et al.*, 1999b; Hill-Kapturczak *et al.*, 2003f). Hypoxia and interferon γ (INF γ) activates the mouse *ho-1* gene but these treatments repress the human *ho-1* gene (Lee *et al.*, 1997; Shibahara *et al.*, 2002a; Kitamuro *et al.*, 2003b). In the mouse *ho-1* gene the enhancer regions E1 and E2

are solely responsible for *ho-1* induction by heme and cadmium while in the human *ho-1* gene in addition to the enhancer regions A and B, the internal region of *ho-1* gene also plays an active part in HO-1 activation in response to heme and cadmium treatments (Hill-Kapturczak *et al.*, 2003a). In response to oxidized lipids, E1 regulates the mouse *ho-1* gene while in the human *ho-1* gene the region between -9.1kb to -11.6kb regulates the *ho-1* gene (Hill-Kapturczak *et al.*, 2003g).

Figure 6

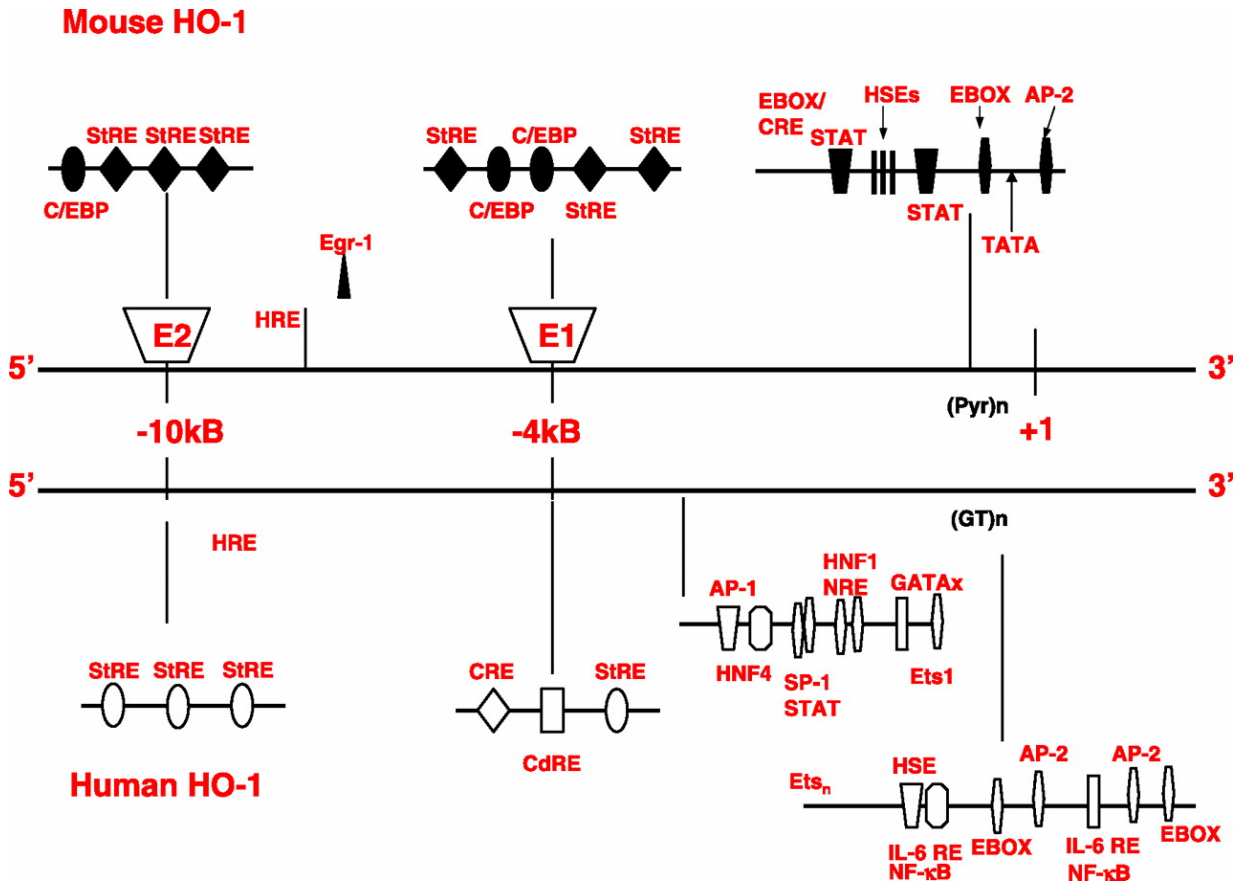


Figure 6 Diagrammatic representation of the response elements in the mouse and human HO-1 promoter (Figure adapted from Ryter *et al.*, 2006)

1.3 The Maf regulatory network

In vitro studies have shown that the small Maf proteins can bind at the MARE sites and regulate the *hmox-1* gene. The name Maf was derived from its structural similarity with its founding member, the v-Maf oncoprotein (Johnsen *et al.*, 1996), which was identified as the transforming gene of a tumor virus isolated from spontaneous musculoaponeurotic fibrosarcoma in chicken. v-Maf was originally discovered as the transduced transforming component of avian Musculo Apo neurotic Fibrosarcoma virus, AS42 (Johnsen *et al.*, 1996). Members of Maf family proteins can be divided into two subgroups, the large Maf proteins, c-Maf (Marini *et al.*, 1997b), MafB (Massrieh *et al.*, 2006a) and NRL (Swaroop *et al.*, 1992), and the small Maf proteins, MafK (Fujiwara *et al.*, 1993a), MafF (Fujiwara *et al.*, 1993b) and MafG (Kataoka *et al.*, 1995a). The large Maf proteins contain a distinctive acidic domain which enables transcriptional activation, while the small Maf proteins lack this acidic domain (Motohashi *et al.*, 1997b). All the Maf proteins have structural similarity and they have a relatively well-conserved basic leucine zipper (b-Zip) domain and the adjacent extended homology region (EHR). This basic region mediates DNA binding to the MARE site and the leucine zipper is responsible for dimer formation with other bZip factors. The large Maf proteins are involved in cellular differentiation and in the regulation of tissue-specific gene expression (Blank and Andrews, 1997a; Kataoka, 2007). The small Maf proteins are about 18 kDa in size (Motohashi *et al.*, 1997a; Blank and Andrews, 1997b) and have been shown to localize in the nucleus. (Francastel *et al.*, 2001; Brand *et al.*, 2004; Massrieh *et al.*, 2006b).

At MARE sites, Maf proteins can form homodimers and repress activation of β -globin (Marini *et al.*, 1997a), NQO1, GST Ya, and g-GCS genes (Wild *et al.*, 1999b; Dhakshinamoorthy and Jaiswal, 2000; Nguyen *et al.*, 2000b). Small Maf proteins can also form heterodimers with a large range of CNC-bZip family transcription factors including nuclear factor erythroid 2 (NF-E2) (Li and Jaiswal, 1992; Prestera *et al.*, 1993), NF-E2-related factor (Nrf)1 (Johnsen *et al.*, 1996; Toki *et al.*, 1997; Marini *et al.*, 1997a), Nrf2 (Andrews *et al.*, 1993; Venugopal and Jaiswal, 1996a; Alam *et al.*, 1999), Nrf3 (Kobayashi *et al.*, 1999a), BTB and CNC homology (Bach) proteins (Oyake *et al.*,

1996), FosB protein (Kataoka *et al.*, 1995b; Shimokawa *et al.*, 2005b). Interestingly the small Maf proteins do not bind with the MafB, v-Maf and Jun proteins (Kataoka *et al.*, 1995c; Shimokawa *et al.*, 2005a). Recent *in vitro* studies suggest that small Maf proteins can also interact with homeobox-leucine zipper protein (Hox12) and mesoderm-specific homeodomain protein (Mhox) but the functional significance of these interactions is still unknown (Kataoka *et al.*, 2001c). The binding of the small Maf proteins with Nrf2 and Bach1 is particularly involved in *hmox-1* regulation and will be the focus of the current study.

1.4 Nrf2

1.4.1 Overview

Three members of the NF-E2 related family (Nrf) of proteins have been identified which are designated Nrf1, Nrf2, Nrf3 (Chan *et al.*, 1993b; Moi *et al.*, 1994b; Kobayashi *et al.*, 1999b). All the NF-E2 related family of proteins share evolutionarily well conserved CNC-like bZip motifs. Nrf1 was the first protein isolated from a cDNA library by using the MARE site of the beta globin locus control region (Chan *et al.*, 1993a; Moi *et al.*, 1994a). Soon after that, Nrf2 was isolated as a NF-E2 binding motif. All the Nrf proteins express ubiquitously in human and mouse cells. It has been demonstrated that Nrf2 is a very potent inducer of Phase 2 proteins compared with Nrf1 and Nrf3 proteins (Venugopal and Jaiswal, 1996b).

The involvement of Nrf2 in activation of cytoprotective enzymes was first demonstrated in a study of regulation of NQO1 (Venugopal and Jaiswal, 1996a). In this first study it was demonstrated that over-expression of Nrf2 cDNA activates the NQO1 gene following various xenobiotics and antioxidant treatments. A significant decrease in expression of NQO1 in the Nrf2 deficient mice further confirmed the role of Nrf2 in NQO1 expression (Venugopal and Jaiswal, 1996a). Later, the role of Nrf2 in activation of

the other cytoprotective proteins including GST Ya, g-GCS, and HO-1 (Alam *et al.*, 1999; Wild *et al.*, 1999a; Nguyen *et al.*, 2000a) has been proposed. An understanding of Nrf2 mediated activation of HO-1 is crucial to the current study. Nrf2 mediated activation of *hmx-1* has been demonstrated following a wide array of stimuli including UVA (Hirota A, 2005), arsenite, cadmium, heme, curcumin (Balogun *et al.*, 2003; Rushworth *et al.*, 2006) hypoxia (Cho *et al.*, 2002) tertbutylhydroquinone (t-BHQ) (Alam *et al.*, 1999; Alam *et al.*, 2000), 15-deoxy-delta12,14-prostaglandin J2 (PGJ2) (Gong *et al.*, 2002) acrolein (Wu *et al.*, 2006), cobalt (Gong *et al.*, 2001; Shan *et al.*, 2006), epigallocatechin-3-gallate (EGCG) (Wu *et al.*, 2005), gold (Kataoka *et al.*, 2001), 1-chloro-2,4-nitrobenzene (CDNB), diethyl maleate (DEM), glucose oxidase (GO) and H₂O₂ (Ishii *et al.*, 2000).

1.4.2 Nrf2 structure and regulation

Nrf2 has six inter-species conserved Neh (Nrf2-ECH homology) domains (Itoh *et al.*, 1999a; Katoh *et al.*, 2001; McMahon *et al.*, 2004). The Neh1 domain contains the CNC-bZIP domain which helps in binding with other dimerization partners and DNA binding. The Neh2 is the N-terminal 100 amino acids domain which contains the DIDLID element and the ETGE tetrapeptide motif and negatively regulates Nrf2 activity (Itoh *et al.*, 1999a; McMahon *et al.*, 2004, McMahon *et al.*, 2006). The detailed mechanism of Neh2-mediated Nrf2 down-regulation is unknown. The Neh4 and Neh5 regions bind with the co-activator CREB (c-AMP-response element-binding protein)/ATF4 and activate transcription (Katoh *et al.*, 2001). The Neh6 domain is redox-insensitive and is involved in Nrf2 degradation (McMahon *et al.*, 2004).

It has been shown that Nrf2 activity is regulated by the cytoplasmic protein Keap1 (Kelch-like ECH-associated protein1). Keap1 possesses a BTB/POZ domain (broad complex, tramtrack, bric-a-brac/poxvirus, zinc finger) and a Kelch domain. The BTB/POZ domain of Keap1 is involved in protein interactions and the Kelch domain is known to bind with the actin skeleton in the cellular cytosol. Under normal relaxed condition Keap1 interacts with Nrf2 and functions as an anchor to keep Nrf2 in the

cytoplasm(Kang *et al.*, 2004). Recently it has been shown that Keap1 promotes Nrf2 degradation by the ubiquitin (Ub)-proteasome proteolysis system(nkova-Kostova *et al.*, 2002;McMahon *et al.*, 2003;Nguyen *et al.*, 2003;Kang *et al.*, 2004). Keap1 engages a subunit of the E3 ligase Cullin3 complex to Nrf2 (Zhang and Hannink, 2003;Kobayashi *et al.*, 2004;Cullinan *et al.*, 2004). Therefore, Keap1 controls total turnover of Nrf2 protein both by balancing its rate of synthesis and degradation (Nguyen *et al.*, 2003) and by cytoplasmic retention. In addition, Keap1 carries multiple cystein residues which are the target of electrophilic attack by various stress agents and also function as a sensor for its antioxidant defence mechanism (Itoh *et al.*, 1999b;nkova-Kostova *et al.*, 2001;Dinkova-Kostova AT, 2002;Wakabayashi *et al.*, 2004). It has been shown that under oxidative stress conditions, Nrf2 is released from Keap1 and migrates to the nucleus. Accumulation of Nrf2 protein in the nucleus is enhanced by stabilisation of the protein. Nrf2 is a short lived protein with an average half-life of 30 min. It has been demonstrated that heme(Alam *et al.*, 2003), and cadmium(Stewart *et al.*, 2003) treatments increase the half-life of the protein from 13 min to around 100 min. The increased nuclear concentration of Nrf2 enhances binding to the small Maf family of proteins and these heterodimers occupy the MARE sites of the target genes in order to activate them (Itoh *et al.*, 1999c). Recently it has been proposed that, following activation of the target genes, Keap1 may transiently migrate to the nucleus and be involved in the degradation of Nrf2 (Nguyen *et al.*, 2005).

1.5 Bach1

1.5.1 Overview

To date two members of the Bach family of proteins, Bach1 and Bach2 have been identified as binding partners for small MafK proteins at the MARE sites (Oyake *et al.*, 1996). Human Bach1 encodes a protein consisting of 736 amino acid residues with a calculated molecular mass of 82.0 kDa (Oyake *et al.*, 1996). Detailed amino acid sequence analysis revealed that Bach1 protein has a 38% sequence homology with the Bach2 protein (Blouin *et al.*, 1998;Ohira *et al.*, 1998). Human *bach1* is located at chromosome 21q22.1 (Blouin *et al.*, 1998;Ohira *et al.*, 1998) and has around 78% sequence similarity with mouse *bach1*. Bach1 expresses ubiquitously in human cell-types including colon, testis, brain, lung, heart, pancreas, spleen, thymus, leukocyte, prostate, skeletal muscle and placenta (Oyake *et al.*, 1996;Blouin *et al.*, 1998;Ohira *et al.*, 1998;Sun *et al.*, 2002). Higher levels of Bach1 protein have been observed in hematopoietic organs; mainly bone marrow and fetal liver (Igarashi *et al.*, 1998a). Bach2 is mainly present in monocytes and neuronal cells (Oyake *et al.*, 1996;Muto *et al.*, 1998).

1.5.2 Functional Significance

The Bach1 protein does not contain a transcriptional activation domain in its protein structure and when transiently transfected together with MafK protein in to fibroblasts appears to work as a repressor complex (Oyake *et al.*, 1996). It has been shown that Bach1/MafK dimers bind to the MARE sites of the HO1 promoter and this leads to repression of HO-1 promoter-reporter activity (Oyake *et al.*, 1996). Along with HO-1 regulation, Bach1 also plays an active role in the regulation of locus control regions (LCR) of the globin genes. Similar to the HO-1 gene promoter, LCR contain multiple MARE sites. It has been demonstrated that Bach1/MafK bind to the MARE sites in the locus control region of alpha and beta globin regions and represses the genes (Igarashi *et al.*, 1998b;Igarashi, 1998b;Igarashi, 1998c;Tahara *et al.*, 2004a). Recently Bach1 mediated repression has been demonstrated in other detoxifying genes including GSH

and NQO1 in different cell models (Dhakshinamoorthy *et al.*, 2005). Most recently Bach1's role in the repression of both types of the iron storage protein Ferritin; Ferritin H and Ferritin L has been suggested (Hintze *et al.*, 2007a). The ferritin gene also carries ARE/MARE type sequences in the promoter regions where Bach1/MafK heterodimers can bind.

In normal unstimulated conditions, Bach1 repressor complex appears to be dominant and prevents access to the transcription activation domains by binding with the small Maf proteins at the MARE sites (Sun *et al.*, 2004a). By doing so Bach1-mediated repressor complex suppress transcription of the target genes so that constitutively low levels of the target proteins are maintained. Bach1's role in HO-1 gene repression was further confirmed in Bach1 deficient mice. In the Bach1 knock-out mice, HO-1 expression is very high in various organs including thymus, intestine, liver, lung and heart (Sun *et al.*, 2002). Further to this, in the mice with *bach1*^{-/-} and *nrf2*^{-/-} genotypes, higher basal levels of HO-1 was observed in thymus which suggested the possibility that the higher basal level of HO-1 is not due to higher expression of Nrf2 but to the absence of Bach1 (Sun *et al.*, 2002). Therefore the general hypothesis for HO-1 gene activation centres around de-repression of Bach1 (Ogawa *et al.*, 2001e; Tahara *et al.*, 2004b; Shan *et al.*, 2006; Ghaziani *et al.*, 2006; Reichard *et al.*, 2007; Abate *et al.*, 2007; Hintze *et al.*, 2007b; Hou *et al.*, 2008).

The importance of Bach1 mediated repression has been demonstrated in various animal models. In the *Bach1*^{-/-} mice, higher levels of HO-1 expression provided significant protection against myocardial ischemia/reperfusion (I/R) injury compared to control mice (Yano *et al.*, 2006). Further to this, over-expression of Bach 1 resulted in megakaryocytic impairment *in vivo* and therefore a role of Bach1 in megakaryocytic lineage cells has been proposed (Toki *et al.*, 2005a). Recently, the role of Bach1 in inflammation and atherosclerosis has been proposed (Omura *et al.*, 2005a). It has been shown that progression of atherosclerosis is significantly reduced in *bach1*-deficient mice in the cuff injury model (Omura *et al.*, 2005b). Over-expression of Bach1 causes bone marrow fibrosis, therefore involvement of Bach1 in the regulation of the bone marrow micro-

environment has been proposed (Toki *et al.*, 2005b). An *in vitro* study revealed that increased expression of Bach1 represses HO1 expression following hypoxia, desferrioxamine (DFO) and interleukin γ treatment conditions in HUVEC cells, human glioblastoma cells (T98G) and human lung cancer cells (A549) (Kitamuro *et al.*, 2003a). It is noteworthy that these treatment conditions repress HO1 expression. In immortalized rat fibroblasts (RAT-1) and human lung cancer cells (Mv1Lu) it has been shown that exposure to cigarette tar causes an early induction followed by a late repression of the HO-1 protein via Bach1 activation (Palozza *et al.*, 2006).

The functional significance of Bach1 mediated HO-1 repression has been proposed in several recent studies. It is known that although by-products of the HO-1 reaction have many beneficial effects, it is important to maintain optimum concentrations of these products by HO-1 repression since increased accumulation of these products in the cellular environment can be toxic. For example, very high concentrations of bilirubin are responsible for diseases such as neonatal jaundice and kernicterus (Dennerly *et al.*, 2001). Free iron can be extremely reactive and can generate various ROS via the Fenton reaction while CO can potentially poison heme proteins and stimulates generation of free radicals (Zhang and Piantadosi, 1992). In addition, heme catabolism via HO-1 requires 3 moles of oxygen and 4 moles of NADPH, so that the energy cost to the cell of continuous HO-1 catabolic activity is high (Shibahara *et al.*, 2003b). Therefore, Bach1 mediated HO-1 repression may also help cells to conserve energy. It is hypothesised that Bach1 mediated HO-1 repression may also lead to a transient increase in the intracellular heme level which is very important for transcriptional regulation of many genes (Sassa and Nagai, 1996). Therefore Bach1 mediated repression generates a feedback loop.

Repression of HO-1 can be potentially very important in cancerous cells. Since HO-1 and its by-products have well documented anti-apoptotic and pro-proliferative roles, HO-1 may contribute to survival and growth of cancerous cells. In addition, the HO reaction is involved in iron homeostasis and iron is essential for proliferation of cancerous cells. Repression of HO-1 may therefore prove beneficial in cancer therapy. Iron production by the HO reaction can provide essential nutrients required for the survival of certain

pathogens, such as bacteria and protozoa, and so can exacerbate harm to a host in infectious diseases (Shibahara *et al.*, 2003a).

1.5.3 Bach1 protein structure and regulation

The structural uniqueness of the Bach 1 protein (Figure 7) has generated interest in its functional role. The Bach proteins contain well-conserved basic leucine zipper domains that are most closely related to the Cap'n'collar (CNC)-type bZip (residues 527–619) domain (Oyake *et al.*, 1996). *In vitro* experiments suggest that the basic region of Bach1 is responsible for DNA binding and the CNC-type bZip domain is responsible for homodimer/heterodimer formation with other bZip containing proteins (Oyake *et al.*, 1996). Along with the bZip domain, Bach proteins contain BTB/POZ domain (broad complex, tramtrack, bric-a-brac/poxvirus, zinc finger) in their amino termini (1-122 amino acid) which helps it to bind with other BTB containing proteins (Zollman *et al.*, 1994; Bardwell and Treisman, 1994; Oyake *et al.*, 1996). Most proteins with BTB domains have been associated with zinc finger motifs (Bardwell and Treisman, 1994; Zollman *et al.*, 1994) while Bach1 was the first protein found in which the BTB domain is associated with bZip domains (Oyake *et al.*, 1996). This unique characteristic was the origin of the Bach terminology (BTB and CNC homology) (Oyake *et al.*, 1996). Bach1 can bind with other Bach1-MafK heterodimers through their BTB domains and form a DNA loop *in vitro*. By forming DNA loops in the promoter of the target gene, Bach1 generates multimeric and multivalent DNA binding complexes (Igarashi, 1998d; Yoshida *et al.*, 1999; Yoshida and Igarashi, 1999) and architect repression of the target genes. By forming DNA loops in the promoters, Bach1 mediates the communication between cis-acting elements that are normally separated from one another and the target genes (Yoshida *et al.*, 1999; Yoshida and Igarashi, 1999). In addition, by binding to multiple MARE sites at two separate locations Bach1 increases the specificity and the stability of protein-DNA complex (Yoshida *et al.*, 1999; Yoshida and Igarashi, 1999).

Figure 7

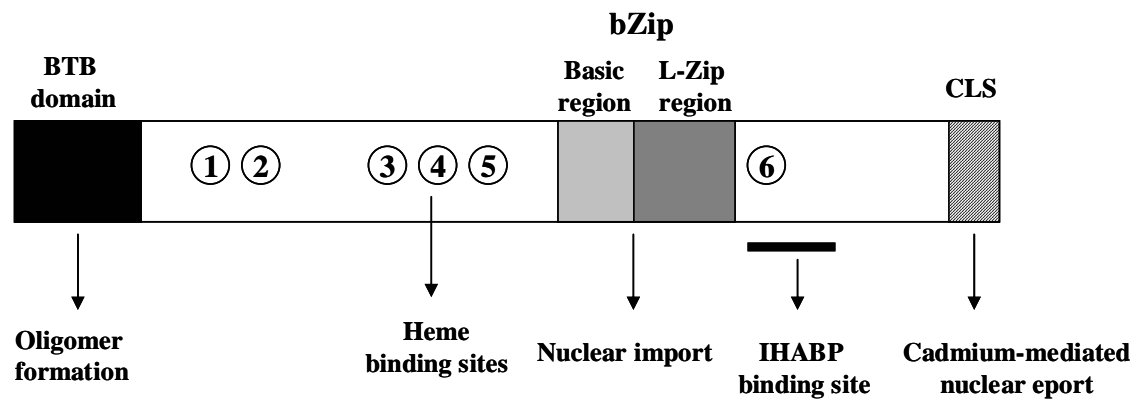


Figure 7 Bach1 protein structure: The unique structure of Bach1 carries combination of bZip domain and BTB domain (broad complex, tramtrack, bric-a-brac). Bach1 has six potentially heme-binding motifs. The C-terminal region of Bach1 carries cytoplasmic localization signal (CLS) region which is responsible for cadmium-mediated nuclear export of Bach1. Bach1 also has a binding site for the intracellular hyaluronic acid binding protein (IHABP).

1.5.4 Bach1 as a heme binding protein

During the initial isolation of Bach1 it was noted that the Bach1 fusion recombinant protein was brownish in colour (Oyake *et al.*, 1996). Later Ogawa *et al.*, demonstrated that the brown colour was due to heme binding (Ogawa *et al.*, 2001d). Bach1 was the first heme-binding protein to be discovered in humans. The Bach1 protein structure analysis revealed that the Bach1 protein has six potential heme binding motifs which are called Cystine-Proline motifs (CP motifs) or heme regulatory motifs (HRM) (Figure 7) (Ogawa *et al.*, 2001c). Detailed analysis of the heme – Bach1 binding revealed that Bach1 binds directly with 5 molecules of heme *in vitro*. Heme reduces the DNA binding capacity of Bach1 and titrates out Bach1 from the MARE sites (Ogawa *et al.*, 2001b). The detailed mechanism behind these phenomena is still not clear. Deletion analysis of the individual

CP motifs revealed that CP motifs in the C-terminal region (CP3-6) are particularly important for heme binding. Deletion of CP3-6 of Bach1 protein abolishes heme binding (Suzuki *et al.*, 2004b). Studies suggest that concentrations of heme as low as 0.03 μ M result in a slight inhibition of Bach1 DNA binding, while 1 mM heme concentrations caused almost complete inhibition of Bach1 DNA-binding activity (Ogawa *et al.*, 2001a). Along with reducing the DNA binding capacity of Bach1, heme induces nuclear export of Bach1 (Suzuki *et al.*, 2004a). Inhibition of heme synthesis in cells prevents the heme mediated nuclear export of Bach1 and treatment with extra-cellular heme promotes the nuclear export. A recent report suggests that heme may bind with the Bach1 protein in two distinct patterns (Hira *et al.*, 2007a). Biochemical analysis of the heme-Bach1 binding revealed two separate peaks in the Raman spectra. Further analysis confirmed the two separate types of heme-Bach1 binding (Hira *et al.*, 2007b). The functional significance for the two separate types of heme bindings with Bach1 is unknown.

Recently it has been shown that heme also regulates total turn-over of Bach1 protein by promoting polyubiquitination and rapid degradation (Zenke-Kawasaki *et al.*, 2007). Extracellular hemin treatment resulted in significantly low levels of endogenous Bach1 proteins while treatment with succinyl acetone, an inhibitor of heme synthesis, resulted in Bach1 protein accumulation in murine embryonic fibroblasts and murine erythroleukemia cells (Suzuki *et al.*, 2004c). By analogy with heme-iron regulatory protein 2 binding, E3 ubiquitin-protein ligase (HOIL-1) binds to heme-bach1 and stimulates the polyubiquitination of Bach1 (Zenke-Kawasaki *et al.*, 2007). Expression of dominant-negative HOIL-1 in murine erythroleukemia cells increases the stability of endogenous Bach1, so that a role of HOIL-1 in heme mediated Bach1 protein degradation has been proposed (Zenke-Kawasaki *et al.*, 2007).

1.5.5 Cadmium-mediated Bach1 export

Along with extra-cellular heme treatment, cadmium also promotes nuclear export of Bach1 (Suzuki *et al.*, 2003c). Cadmium-mediated Bach1 nuclear export arises via a different mechanism. Bach1 carries cytoplasmic localisation sequence (CLS) in its C-terminus which have homology with the evolutionarily well conserved nuclear export signal (NES)(Hoshino *et al.*, 2000). Cadmium treatment activates these CLS sequence and promotes nuclear export of Bach1 via the Crm1/Exportin pathway (Suzuki *et al.*, 2003b). It has also been suggested that more than one factor is involved in the cadmium-mediated export. The BTB/POZ region of the Bach1 protein is well known to be involved in protein/protein interactions and this region may interact with other cytoplasmic proteins containing BTB domain in order to facilitate the export (Igarashi, 1998a). Igarashi and colleagues have suggested that CLS may not be the only factor responsible for cadmium-mediated export of Bach1 and have proposed that MAPK pathways may be involved. Cadmium-mediated activation of ERK1/2 is involved in the Bach1 subcellular localisation and inactivation of Bach1 activity (Suzuki *et al.*, 2003a). Both heme- and cadmium-mediated Bach1 nuclear export are mediated by the nuclear membrane transport protein Crm1. Inhibition of Crm1 protein by its specific inhibitor, Leptomycin B (LMB), prior to treatment with these agents prevented the nuclear export.

Sub-cellular localisation of Bach1 protein is further regulated by the intracellular hyaluronic acid binding protein (IHABP). Recently it has been suggested that Bach1 protein can interact with IHABP in the cytoplasm(Yamasaki *et al.*, 2005). IHABP is the cytoplasmic microtubule associated protein which can bind with Bach1 in between the CLS and bZip regions. It has been proposed that many human cells express the alternative spliced variant Bach1t which lacks these IHABP binding region and which always stays in the nucleus(Kanezaki *et al.*, 2001). The detailed functional significance of Bach1 expression and IHABP binding is still not clear.

1.6 Aims and Objectives

This study will determine the role of Bach1 in the UVA-mediated regulation of the HO-1 gene in primary human skin fibroblasts. To achieve this, we will first determine the effect of UVA irradiation on the Bach1 gene in the primary human dermal fibroblast cell line FEK4. The dose-dependant effects of UVA irradiation on Bach1 protein levels will be monitored. Basal and induced levels of Bach1 expression will be investigated in human skin cells. It is known that the localisation of negative regulatory proteins influences HO-1 transcriptional regulation, therefore this study will determine the time-dependant effects of UVA irradiation on the localisation of Bach1 protein. We will study events related to UVA-mediated activation of the HO-1 gene in order to establish the relationship between Bach1 and Nrf2 expression and localisation, and the HO-1 protein induction. Since heme influences the expression of both the activator and the repressor protein, the role of heme in modulation of Bach1 sub-cellular localisation and its effects on HO-1 expression will be identified by inhibiting heme synthesis in the cells prior to UVA treatment by treatment with succinyl acetone. The role of the nuclear membrane transport protein Crm1 in the sub-cellular localisation of Bach1 protein will be identified.

The role of Bach1 in basal and UVA-inducible expression of HO-1 will be determined in FEK4 by using recombinant plasmids to express Bach1 and MafK proteins. To confirm the involvement of Bach1 protein in HO-1 repression, RNAi techniques will be employed. We will develop efficient and cost effective methods to produce Bach1 and HO-1 specific siRNAs. The Bach1 specific siRNA will be used to silence the gene and its effect on basal and UVA-mediated HO-1 expression will be studied. We will study the basic mechanism underlying down-regulation and refractoriness to re-induction that occurs following a second UVA dose. We will measure Bach1 and Nrf2 proteins levels for up to 72h and following a second UVA dose to establish the relationship between Nrf2 and Bach1 protein levels and localisation and the development of refractoriness of HO-1 gene to re-induction by UVA irradiation.

2. Materials and Methods

2.1 Chemicals

Routine mammalian cell culture materials were purchased from GIBCO Invitrogen Ltd. All chemicals were of analytical grade and purchased from Sigma Chemicals, UK unless stated otherwise.

2.2 Cell storage and maintenance

Human skin fibroblasts (FEK4) were stored in liquid nitrogen in medium supplemented with 10 % v/v dimethyl sulphoxide (DMSO). Cells were rapidly thawed and transferred to a centrifuge tube containing 10ml of media. The tube containing cells was centrifuged (1,000 rpm for 5 min) and the supernatant was discarded. The cell pellet was then resuspended in an appropriate volume of culture medium and incubated using the cell culture conditions described in section 2.3.

2.3 Cell Culture

Cells were cultured in EMEM supplemented with 2mM L-Glutamine, 50 u/ml penicillin, 50 µg/ml streptomycin, 0.2% sodium bicarbonate and 15% FCS. Experimental cells were used between passage 8 to 13 and passaged weekly using 0.25% trypsin. Cells were cultured at 37°C in 5% CO₂ / 95% air humidify incubator.

2.4 UVA irradiation

Cell populations grown at 80% confluency were irradiated at room temperature on day 3 using a broad spectrum UVA lamp (350-450 nm, Sellas, Germany). The required time for UVA exposure was quantified using an IL1700 radiometer (International Light, Newbury USA) with SEE400 probe. Before treatment, media was removed and saved for use later as conditioned medium. Cells were rinsed twice with phosphate buffered saline (PBS) (Oxoid Ltd. UK) and covered with PBS/Ca-Mg (0.5µg/ml each of CaCl₂ and MgCl₂). After irradiation, the conditioned media was added back. Controls (Shams) were treated same except they were not irradiated.

2.5 Succinyl Acetone treatment

A stock solution of 100mM 4, 6-dioxoheptanoic acid [Succinyl Acetone (SA)] was prepared in distilled sterilized water and was subsequently filter sterilized. Prior to treatment the media was collected and retained. Cells were washed twice with PBS and were treated with 5mM SA working solution in serum free media for 18-22 h at 37°C in the incubator. Following treatment the conditioned media was added back. The control cells were kept in the incubator throughout the experiment

2.6 LMB treatment

Prior to treatment the media was collected and retained. Freshly confluent FEK4 cells were washed twice with PBS. Cells were treated with 40ng/mL Leptomycin B (LMB) (LC Laboratory) working solution in the conditioned media for 2 h at 37°C in the incubator. The conditioned media was added back following treatment.

2.7 Time Course of mRNA accumulation and RNA extraction

FEK4 (5×10^5 cells/dish) cells were seeded in 10 cm dishes. On day three cells were treated as mentioned above and after treatment 8.5ml of conditioned media was added back and incubated for required time in conditioned media. For Northern blot, after incubation time each dish was washed twice with PBS and 4ml of TRIZOL was added per dish and stored in 14ml Grainer tubes at -80°C until RNA extraction.

2.8 RNA extraction

Total cellular RNA was extracted according to the manufacturer's protocol (Invitrogen life technology). Extracted RNA was 1:100 diluted in DEPC treated water and measured at 260 and 280nm in a double beam spectrophotometer.

2.9 Northern blotting

1% agarose gel containing 14ml of 10x MOPS buffer and 23ml of 37% formaldehyde was prepared in a horizontal electrophoresis tank. Total RNA (15 µg/well) were electrophoresed at 100V for 2.5-3 h. After electrophoresis, gel was rinse twice with sterile water and soaked in alkaline rinse solution (75mM NaOH, 100mM NaCl) for 15

min at room temperature with gentle shaking. The gel was neutralized twice with 100 mM Tris-HCl (pH 7.5) at room temperature for 15 min. Northern transfer was set up in 10x SSC as describe in the Zetaprobe manual.(Bio-Rad Laboratories) After 18-24 h transfer membrane was rinse with 2x SSC. Membranes were baked for 30 min at 80 °C and then pre-hybridized and hybridized at 65°C. Pre-hybridization and hybridization solutions consisted of 7% SDS, 250mM Na₂HPO₄, 1mM EDTA. ³²P-labbelled-cDNA probe, prepared by random priming, was added to the hybridization solution. Purification of probes was carried out on the ELUTIP-d column (Schleicher-Schuell Bioscience). The probe was denatured and added to the hybridization solution. After overnight hybridization, membranes were washed and exposed to phosphorimager screen. The screen was scanned using phosphorimager (Phosphorimager SF, Molecular Dynamics, USA) and analyzed using the computer programme Image Quant, version 3.3. The filters were re-hybridized with a ³²P-labelled probe derived from a 1400-base pair cDNA Pst 1 fragment of the rat glyceraldehyde phosphate dehydrogenase gene (GAPDH) for an internal control of loading between samples.

2.10 Whole cell extracts

Following treatment cells were detached and then pelleted by centrifugation (13,000 rpm for 30 seconds) and the supernatant was discarded. The cell pellets were then lysed by using an appropriate volume of stabilisation buffer [50 mM tris-HCl pH 7.5, 150 mM NaCl, 10 % Glycerol, 5 mM ethylene-diaminetetraacetic acid (EDTA), 1 % NP40, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The cell lysate was then centrifuged at 4°C for 5 min at 13,000 rpm. The supernatant containing total cellular protein was collected and flash-frozen using ethanol bath containing dry-ice and 100 % ethanol. Cell extracts were stored at -20°C.

2.11 Quantification of protein concentration

The Bio-Rad protein assay based on the Bradford dye binding procedure (Bradford, 1976) was used to calculate protein concentration. A standard curve was generated using six concentrations (0 – 12 mg/L) of bovine serum albumin (BSA) in the same solvent as the cell extract. For each sample Bradford reaction was performed in duplicate using 4µl

of extract. The protein concentration was determined using a 96-well spectrophotometric microplate reader (VERSAmax, Molecular Devices) and the software package SoftMaxPro.

2.12 Acrylamide gel preparation

7.5 % Acrylamide gel solution was prepared by adding 2.8 ml of 40% acrylamide (40 %, 29:1, acrylamide: bis-acrylamide), 5.6 ml 1 M Tris pH 8.8, 75 µl of 20 % sodium dodecyl sulphate (SDS) and 6.6 ml MilliQ water. The acrylamide gel was set by adding 40 µl N,N,N',N'-tetramethylethylenediamine (TEMED) and 100 µl of 10% ammonium persulfate in PBS. The gel setting cascade was arranged by using BioRad Mini Western gel system. The gel solution was poured in the empty gel cascade and allowed to set. 50µl of water saturated butanol solution was added on top of the gel solution to make sure an even surface and to avoid evaporation. The gel solution was allowed to set for 15-20min. The stacking gel was prepared using 0.51 ml of 40 % acrylamide (40 %, 29:1, acrylamide: bis-acrylamide), 0.5 ml 1 M Tris pH 6.8, 20 µl of 20 % SDS and 2.86 ml MilliQ water. After adding 50µl ammonium persulfate and 25µl TEMED, the stacking gel solution was poured on top of the set gel. The gel was allowed to set for further 15-20min.

2.13 Western blot analysis

Total protein (30µg/well) were diluted with 3 x loading buffer (180 mM tris pH 6.8, 3 % SDS, 150 mM DTT, 30 % glycerol, 0.0015 % bromophenol blue) and heated to 95°C for 5 min. The samples were loaded on 7.5 % SDS-polyacrylamide gels and electrophoresis was performed at 150 volts for 90 min with running buffer (1.5 % w/v tris, 7.2 % w/v glycine, 0.5 % SDS). Following the electrophoresis the proteins were transferred onto nitrocellulose membranes (Amersham Biosciences, UK). Electro-transfer was performed at 100 volts for 90 min at 4°C in transfer buffer (3 % w/v tris, 14.4 % w/v glycine, 20 % methanol). Following transfer the blots were blocked over-night at 4°C in 5 % milk powder in 0.05% Tween-PBS. Blots were incubated with primary antibody at room temperature for 90min and subsequently incubated with corresponding HRP conjugated secondary antibody (SantaCruz Biotechnology) at room temperature on 3D rocking

platform (Stuart Scientific) for 1h. Each antibody staining steps were followed by three times 10min washing steps in 0.05% Tween-PBS on 3D rocking platform (Stuart Scientific). Proteins were visualized with Enhanced Chemiluminescence (ECL) reagents (Amersham Biosciences, UK).

2.14 Construct preparation

Bach1-GFP and MafK-RFP over-expressing plasmids were prepared by Dr. Steve Mitchell, University of Bath. Inserts were generated from two-step RT-PCR reactions using total RNA isolated from human monocytes. In the first step cDNA pool was generated from total RNA using Random Hexamer system (Invitrogen Bioscience). The inserts were generated using F – 5' ATATAgAATTCATgTCTCTgAgTgAgAACTCgg
R – 5' ATATAggTACCAACTCATCagTAgTACATTTATCag primers for Bach1 and
F – 5' TATATgAATTCATgACgACTAATCCCAAACC
R – 5' TATATACCggTAAGgATgCAGCCgAgAAgg primers for MafK. In the case of Bach1-GFP, the insert was ligated in the region of EcoR1/Kln1 of the parental vector pEGFP-N1 (CLONTECH, UK). In the case of MafK-RFP construct, the amplified PCR product was ligated in the region of EcoR1/Age1 of the parental vector pDsRed1-N1 (CLONTECH, UK). The ligated vectors were transformed in competent *E.coli* JM109 cells (Promega, UK) by electroporation technique. The plasmids were checked for sequence accuracy. The detailed map with main features of both the parental vectors is described in figure 8 and 9.

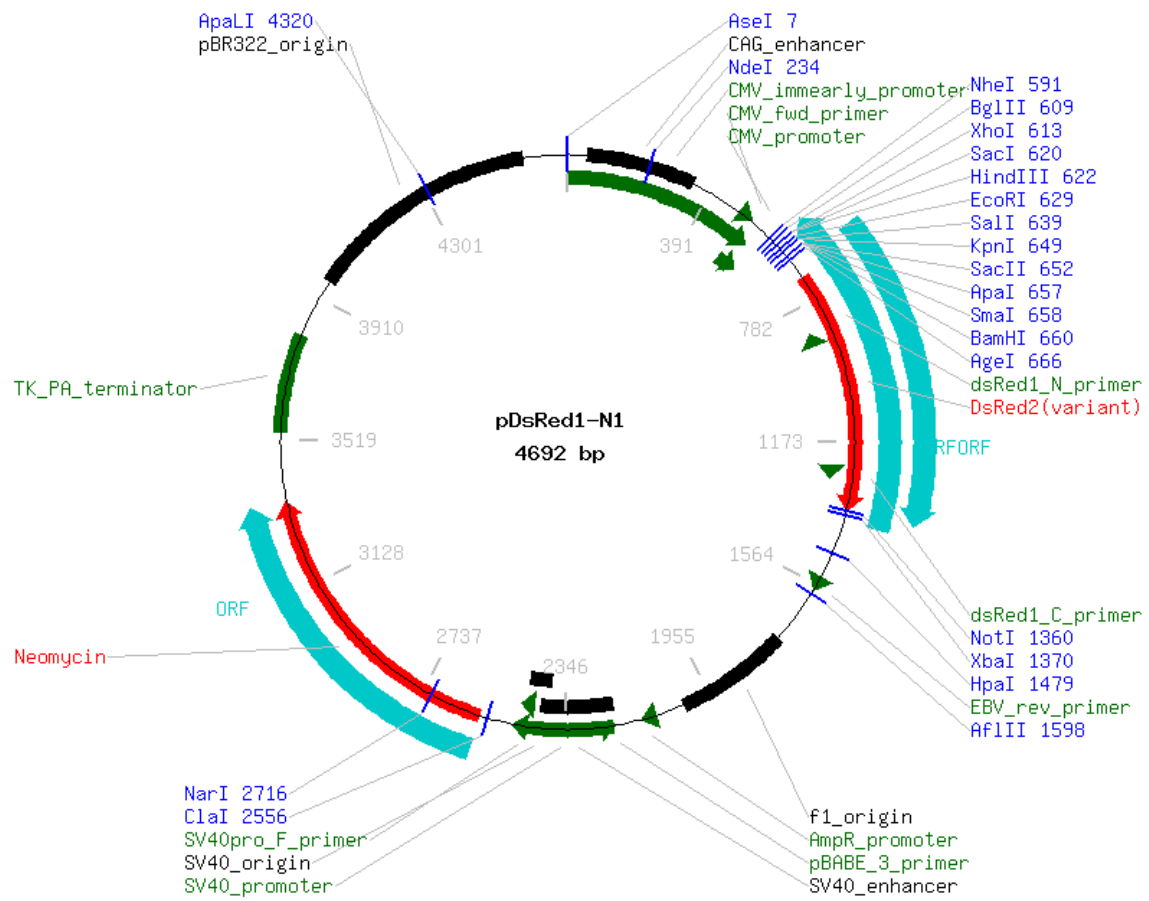


Figure 8 Map of pDsRed1-N1 vector

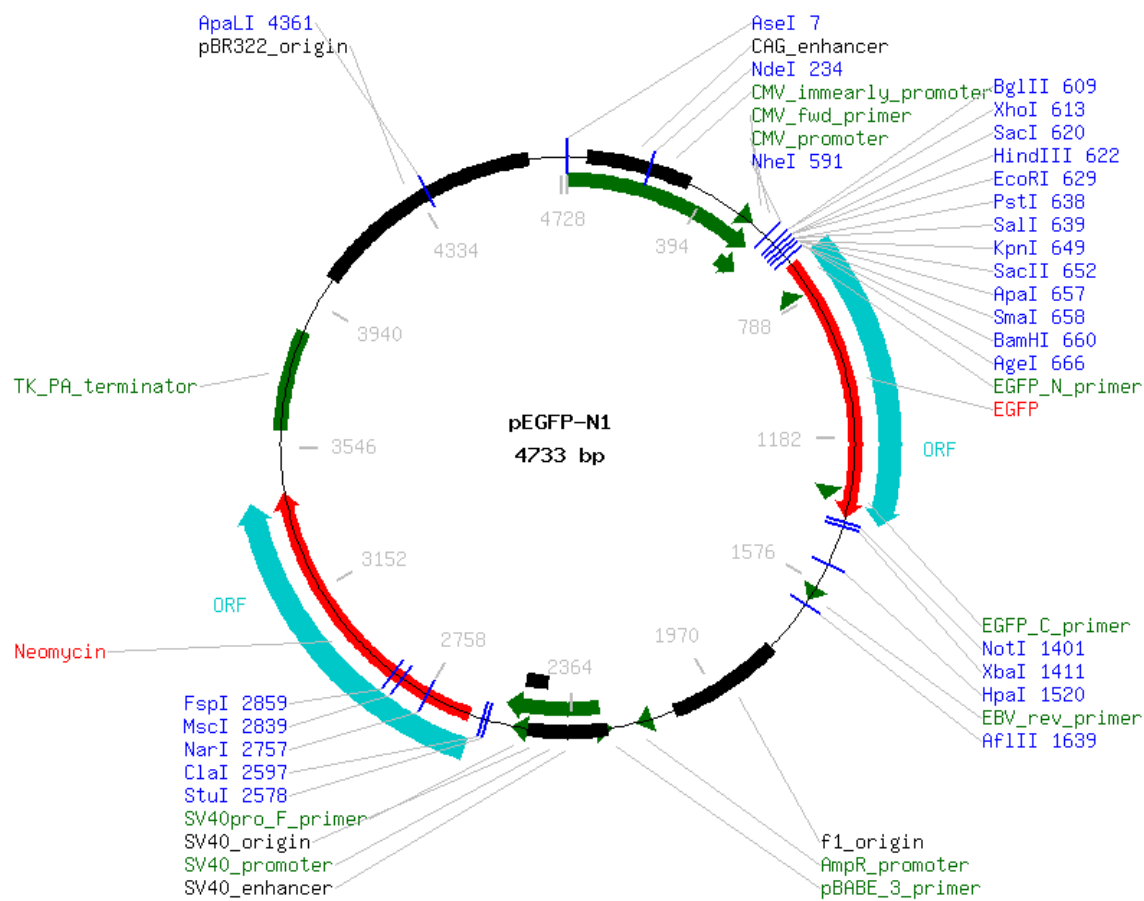


Figure 9 Map of pEGFP-N1 vector

2.15 Transfection

FEK4 cells were grown on coverslips in the 3cm dishes. Before transfection, media was removed and saved for use later as conditioned medium. Cells were washed twice with PBS. The transfection reaction was prepared in Optimem media with either 1.5µg of both Bach1 and MafK constructs or 5nMolar of Bach1 specific siRNA cocktail and 3 ml of Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. Transfection was performed for 4-6 hours in the Optimem media in the incubator at 37°C. Following transfection, the transfection reagent was aspirated and the cells were washed twice with Optimem media and the condition media was added back.

2.16 Immunocytochemistry

Following the indicated treatment, FEK4 cells were washed twice with PBS and fixed with 4% paraform aldehyde(PFA) in PBS for 20 min at room temperature followed by two times wash with PBS at room temperature. The fixed cells were permeabilised by adding ice cold absolute Methanol for 5 min at -20°C. After 5 min, methanol was discarded and cells were rinsed twice with PBS. Blocking of non-specific sites was accomplished by application of 2 – 3 drops of Image-iT™ Fx signal enhancer (Invitrogen Life Technologies) on the cells. The cells were incubated for 30 min at room temperature according to the manufacturer's instructions (Invitrogen Life Technologies). Following blocking step cells were rinsed with PBS. Primary antibody staining was performed with either anti-HO1 (1:200 dilution) (Stressgen, UK), anti-Nrf2 (1:100 dilution) (C-20 Santa Cruz Biotechnology) or anti-Bach1 (1:400 dilution) (C-20 Santa Cruz Biotechnology) antibodies for 1 h at room temperature, followed by twice 5 min wash with PBS on shacking rocker at room temperature. Secondary antibody staining was performed for 1 hour with Alexa Fluor 647 Anti-mouse SFX kit for HO1 and Alexa Fluor 595 Anti-Goat for Bach1 and followed manufacturer's instruction (Invitrogen Life science). The antibodies were diluted in 0.2% BSA in PBS. Each antibody staining was followed by twice 5 min wash with PBS at room temperature. The cells were incubated with nuclear stain, Hoechst stain (Sigma) for 5 min at room temperature, followed by 5 min PBS wash. Detailed information about primary and secondary antibodies' sources and

dilutions is described in Table 1. The coverslips were mounted on the glass slides using Dakocytomation fluorescent mounting medium (DakoCytomation, UK).

Table 1: Antibody information:

Protein	Primary Antibody Source	Application	Dilution	Secondary antibody dilution
Bach1	Santacruz Biotechnology C-20	Immuno cytochemistry	1:400	1:1000 Alexa Fluor 595 Anti-Goat (Invitrogen)
		Western Blot		1:5000 HRP conjugated Anti-Goat (Sigma)
	A1-5	Immuno cytochemistry	1:400	1:1000 Alexa Fluor 595 Anti-Rabbit (Invitrogen)
Nrf2	Santacruz Biotechnology C-20	Immuno cytochemistry	1:100	1:1000 Alexa Fluor 595 Anti-Rabbit (Invitrogen)
		Western Blot		1:5000 HRP conjugated Anti-Rabbit (Sigma)
HO-1	Stressgen	Immuno cytochemistry	1:200	1:1000 Alexa Fluor 647 Anti-Mouse (Invitrogen)
		Western Blot		1:5000 HRP conjugated Anti-Mouse (Sigma)

2.17 Image Acquisition and Quantification:

The images were taken with oil immersion epifluorescence (Zeiss Immersol, 518N) at 43x magnification on a microscope, equipped with a Nikon Eclipse TE2000-U camera, Lambda 10-2 filter unit (Shutter Instrument Co, UK) and UltraVIEW software package. For the quantification of the fluorescence intensity of the images Image J software package was used. The area covering an individual cell was selected and the fluorescence

intensity in the predefined region was measured. Mean fluorescence intensity in the predefined area was determined by dividing total fluorescence intensity by the area in pixels. By using same method mean fluorescence intensity for 300 individual cells were determined for each sample and compared with the mean fluorescence intensity of corresponding controls.

2.18 Statistical Analysis:

Results are expressed as a mean and error bars represent standard deviation above and below the mean. Comparison of means of two groups of data was made using Student paired t-test. A one-way analysis of variance (ANOVA) was used in SPSS software package for Windows to compare means of more than two groups of data. Unless specified, all figures contain data from three independent experiments.

2.19 siRNA preparation targeting HO-1 and Bach1

The siRNA cocktails targeting the open reading frame(ORF) of HO-1 and Bach1 gene were prepared according to the guidance provided in the instruction manual of Silencer™ siRNA Construction kit (Ambion) by using individual components. In order to use a DNA sequence for *in vitro* reverse transcription to generate double stranded RNA (dsRNA) synthesis, the targeted gene sequence should have minimal T7 RNA polymerase promoter recognition sequence (TAATACGACTCACTATAGG) at the 5'end.

The template DNA sequence (targeted ORF of HO1 and Bach1 gene) with flanking T7 recognition sequence, were amplified using PCR. PCR amplification was performed in a 50 µl reaction volume containing, 25µl Ready mix solution (Sigma), 10pmol of primers, 5ng cDNA (HO1 and Bach1), 1.5mM and 2mM MgCl₂ for Bach1 and HO1 respectively. 5'TAATACGACTCACTATAGG CAGTCAGGCAGAGGGTGATAG3' forward primer and 5'TAATACGACTCACTATAGGGGCTTCCCTCTGGGAGTCT3' reverse primer for HO1 and 5'TAATACGACTCACTATAGGAGCCTTTGTCAGGTACAGACG3' forward and 5'TAATACGACTCACTATAGGGCTGCAAATGTCACTCCAGA3' reverse primer for Bach1 were used. Amplification was carried out as mentioned in the

Silencer™siRNA Construction kit. The amplified products were cleaned with QUIquick column (Qiagen) and checked on 2% agarose gel (Figure 10 A).

Long dsRNA was transcribed using minimal T7 RNA polymerase promoter recognition sequence flanked PCR product as a template. The transcription reaction was carried out in 50 µl reaction volume containing 0.5-2 µg template DNA, 200mM of each rNTP (Promega), 2U Inorganic pyrophosphatases (New England Bioscience), 250U T7 RNA polymerase (New England Bioscience). The reaction was carried out for 2-3 h at 37°C. After transcription the remaining template DNA was removed by DNase treatment according to the manufacturer's instructions (Promega). The products were cleaned and check on 2% agarose gel. As expected, the size of DNA template and dsRNA were same (~210bp) (Figure 10, A and B). The dsRNAs were treated with RNaseIII (Ambion) for 1 h at 37 °C. The reaction was stopped by adding 2.5µl EDTA (0.5mM). The siRNA were cleaned by using microcon YM-30 filter units (Amicon). RNaseIII digestion reactions gives a siRNA cocktail of ~12-15bp. The size of the siRNAs was checked on a 15% non denaturing polyacrylamide gel. As shown in Figure 10 the siRNA cocktail appears as a smear of the band in the expected region.

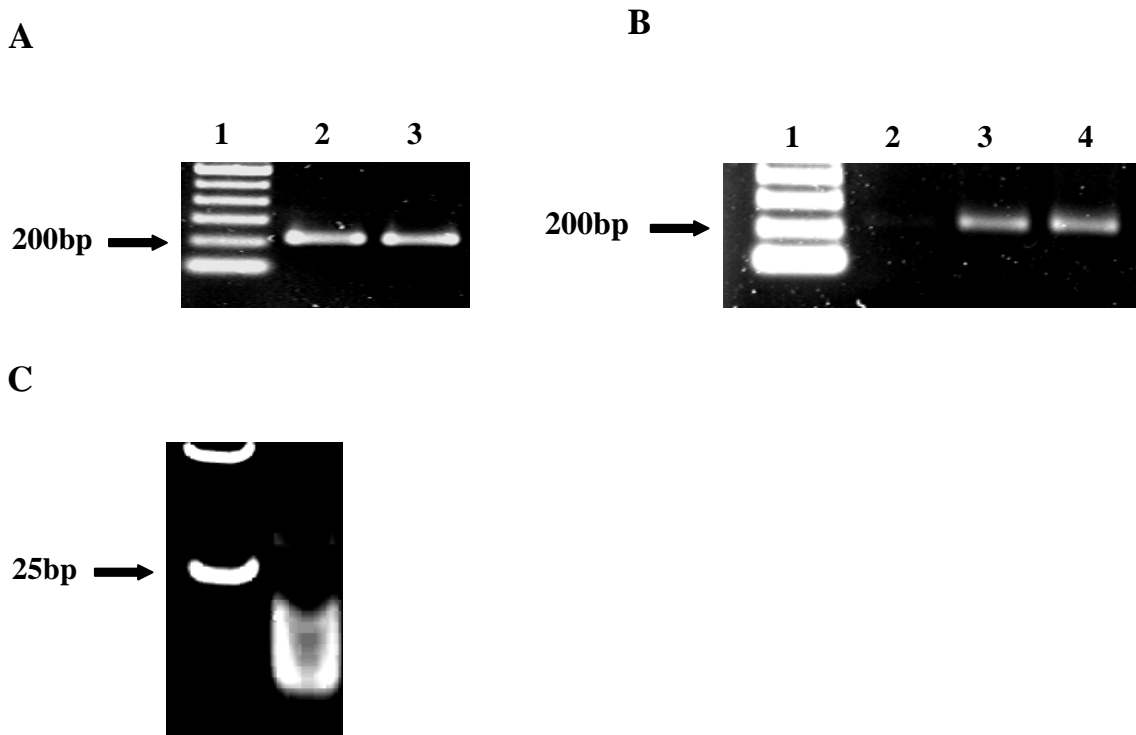


Figure 10 (A) Lane 1 contains a 100bp DNA ladder. Lane 2 contains the targeted DNA of the Bach1 gene, flanked with T7 RNA polymerase promoter recognition sequence. Lane 3 contains the dsRNA transcribed with T7 RNA polymerase by using the Bach1 gene specific DNA sequence as template. (B) Lane 1 contains a 100bp DNA ladder. Lane 3 contains the targeted DNA of the HO-1 gene, flanked with T7 RNA polymerase promoter recognition sequence. Lane 4 contains the HO-1 gene specific dsRNA. Lane 2 is an empty well. (C) Lane 1 contains a 25bp marker. Lane 2 contains Bach1 gene specific siRNA digested with RNaseIII by using Bach1 specific dsRNA.

2.20 Agarose gel electrophoresis

2% agarose gel was prepared by dissolving electrophoresis grade agarose (Invitrogen Life Technologies, UK) in tris-acetate buffer (TAE) (20 mM tris acetate, 1 mM EDTA pH 8.0) in a microwave oven. The gel solution was allowed to set in a horizontal electrophoresis tray (Danaphor, Grandvaux, Switzerland) with a comb. The samples were diluted in 6xloading dye (Invitrogen Life Technologies, UK). Electrophoresis was performed at 55 volts for 60 – 90 min. Following electrophoresis the gel was stained with 0.7 µg/ml ethidium bromide solution at room temperature for 20 min. The nucleic acid bands were visualised under UV light by using Gene genius (SynGene, Synoptics Ltd).

3. Results

3.1 Induction of Bach1 after UVA Treatment:

The effect of UVA treatment on Bach1 mRNA accumulation was examined in primary human skin fibroblasts (FEK4) by Northern blotting. FEK4 cells are primary human skin fibroblasts derived from a human skin explant (Tyrrell and Pidoux, 1986). FEK4 cells were used in all experiments in this study. A moderate dose of UVA radiation (250kJ/m^2) increases the level of Bach1 mRNA accumulation during post-treatment incubation in conditioned media and this reached a peak (3-fold increase) after 4 h (Figure 11).

The effects of UVA irradiation on endogenous Bach1 protein were checked by an Immunocytochemical technique using two different antibodies raised against Bach1 protein, the Bach1 A1-5 antibody (Zenke-Kawasaki *et al.*, 2007b) and Anti-Bach1 (Santacruz). As shown in figure 12, newly synthesized Bach1 protein starts to accumulate in the nucleus by 4 h and is still increasing by 8 h following UVA irradiation. The fluorescence intensity of each image was measured and compared to untreated cells. As shown in figure 13, UVA induces an approximately two-fold induction in the nuclear accumulation of Bach1 8h after UVA treatment as compared to untreated cells. The results were confirmed by using Bach1 A1-5 antibody (Figure 14). The dose-dependant effect of UVA irradiation on Bach1 was checked in FEK4 cells. Freshly confluent FEK4 cells were treated with various doses of UVA. Following the treatment, cells were incubated for 8h at 37°C in conditioned media. As shown in figure 15 and 16, an approximately two-fold induction in the nuclear accumulation of Bach1 was observed in the cells irradiated with a dose of 150, 200, 250 and 500kJ/m^2 of UVA irradiation as compared to untreated cells. Accumulation of endogenous Bach1 protein was also confirmed by western blot analysis. FEK4 cells were treated with a moderate dose of UVA radiation (250kJ/m^2) and incubated in conditioned media at 37°C for 8h. Whole cell protein extracts were collected following incubation and Bach1 protein levels were monitored by using anti-Bach1 antibody as described in the materials and methods

(figure 17). No decrease in Bach1 protein expression was observed after UVA treatment and Bach1 signal was always observed to be predominantly in the cell nucleus. This indicates that UVA does not lead to degradation of Bach1 protein or mediate rapid immediate nuclear export of Bach1 protein as has been reported following heme, cadmium and arsenite treatments(Suzuki *et al.*, 2003a;Suzuki *et al.*, 2004;Reichard *et al.*, 2007).

Figure 11

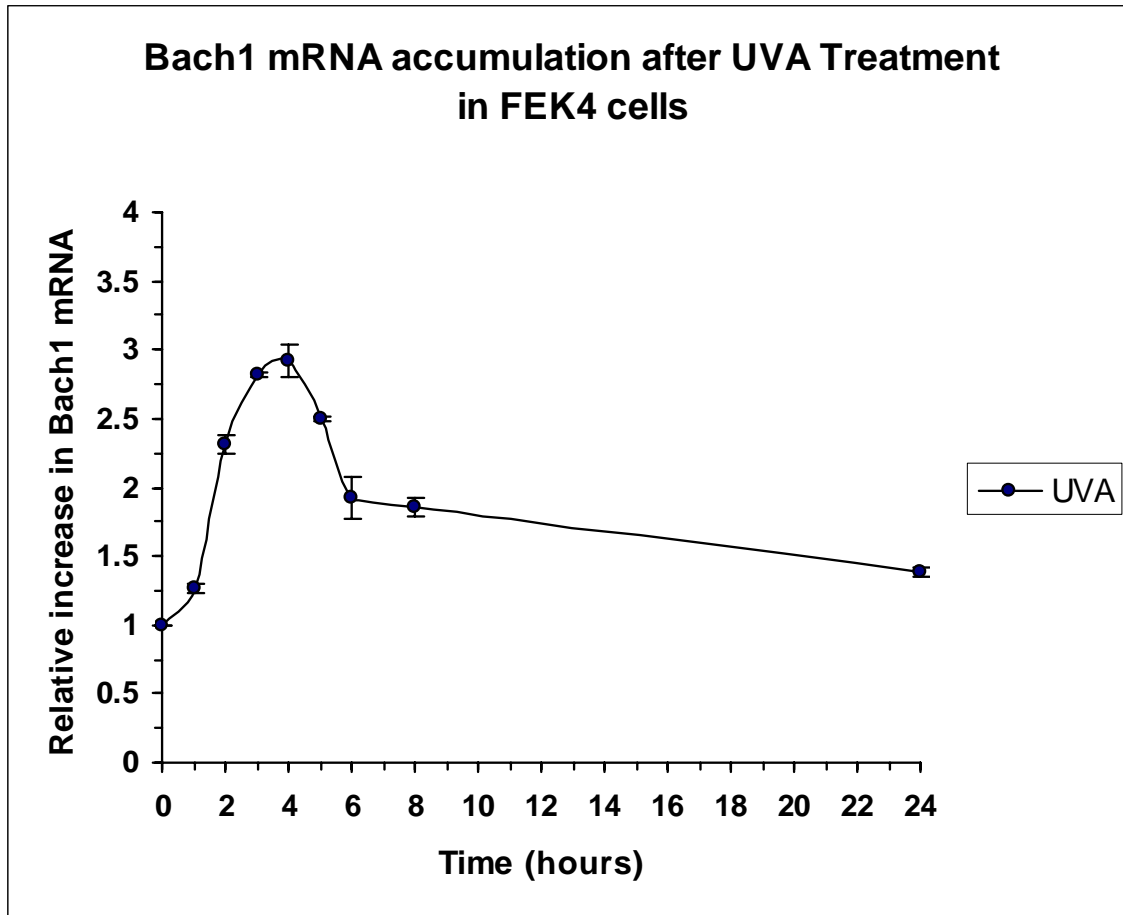


Figure 11 Bach1 mRNA accumulation after UVA treatment: FEK4 cells were treated with a moderate dose of UVA (250kJ/m²) and incubated in conditioned media at 37°C for the indicated time. Total cellular RNA was isolated using TRIZOL after different incubation time. Bach1 mRNA was quantified using Northern blot analysis. GAPDH mRNA levels were determined in all samples and used for normalizing Bach1 mRNA levels. The density of each band was measured using the computer programme Image Quant, version 3.3 and data were expressed as the relative fold increase over untreated samples. The data presented are the mean fold induction for each time point from three independent experiments.

Figure 12

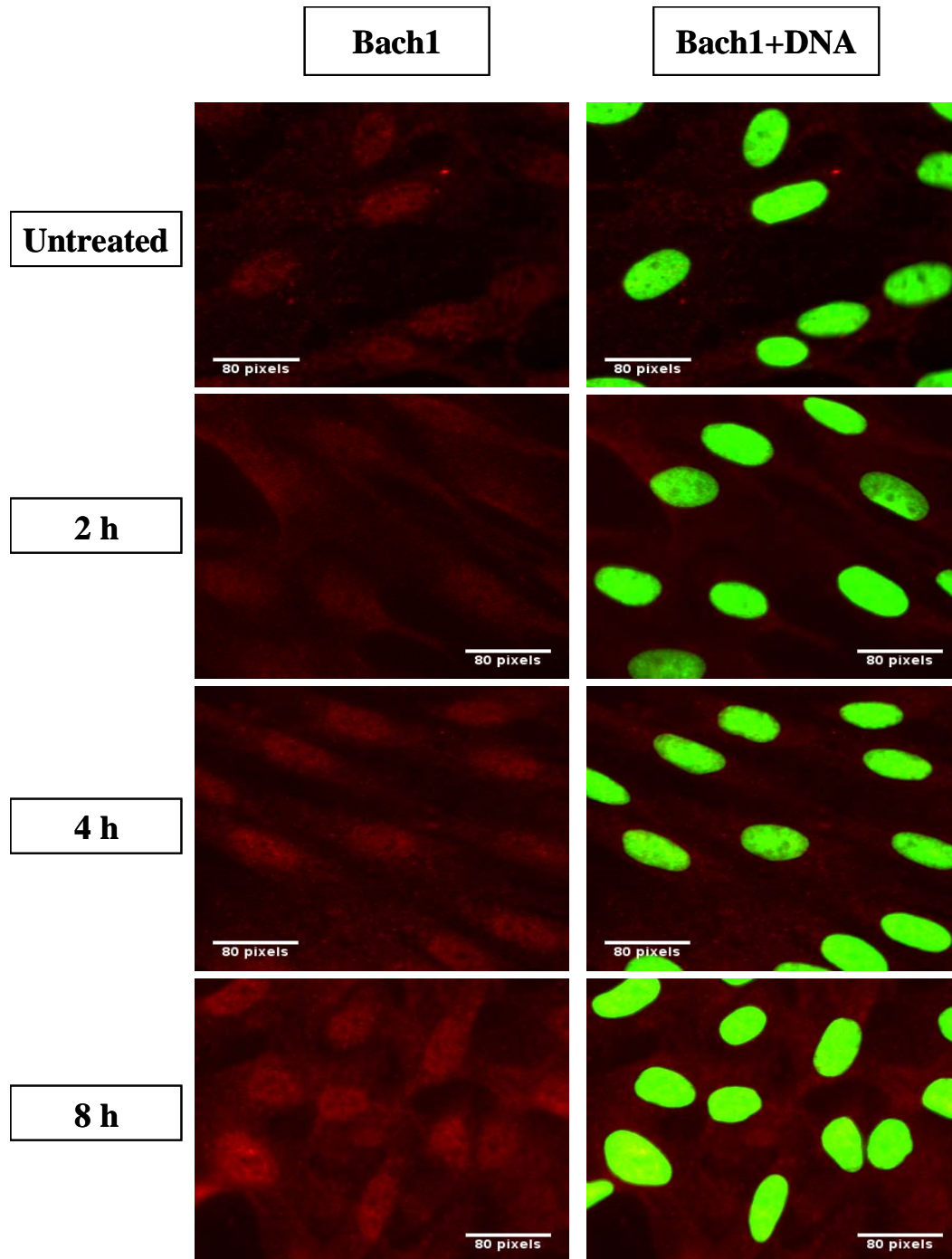


Figure 12 Bach1 protein induction following UVA irradiation: FEK4 cells were grown on cover slips and irradiated with a moderate dose of UVA (250kJ/m²) as mentioned in the materials and methods. The cells were fixed at the indicated time-points with 4% PFA and immuno-staining was performed using anti-Bach1 goat polyclonal primary antibody followed by Alexa Fluor 595 rabbit anti-goat

secondary antibody. The cell nuclei were stained with Hoechst stain. The images for the Bach1 staining (Red colour) and the nuclei (Green colour) were obtained with the 595nm filter and the UV filter settings respectively.

Figure 13

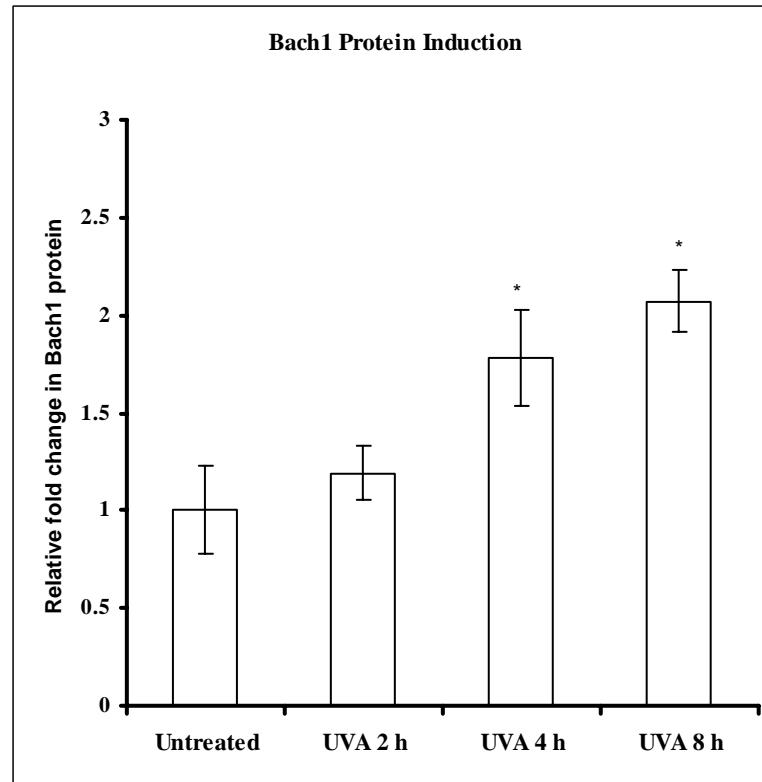


Figure 13 Bach1 protein induction following UVA irradiation: Fluorescence intensity was quantified using the Image J software package. The mean gray value of 300 cells for each condition from three individual experiments was quantified for each sample and data were expressed as the relative fold increase over untreated samples. Data are mean \pm S.D. of 5 independent experiments. The Student paired t-test was used to evaluate the statistical significance ($p < 0.05$) of each data point.

*** Significance level when compared with the untreated control**

Figure 14

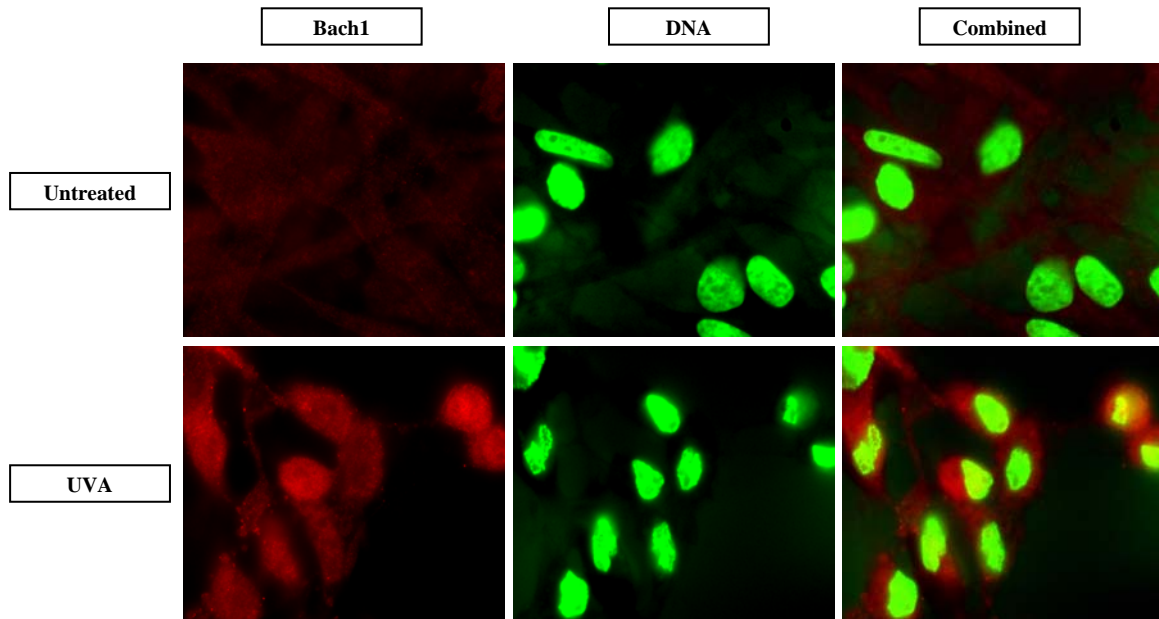


Figure 14 Nuclear accumulation of Bach1 protein following UVA irradiation:FEK4 cells were treated with UVA (250 kJ/m²) and incubated in conditioned media at 37°C for 8h. The cells were fixed with 4% PFA and immuno-staining was performed using anti-Bach1 rabbit polyclonal primary antibody followed by Alexa Fluor 595 goat anti-rabbit secondary antibody. The cell nuclei were stained with Hoechst stain. The images for the Bach1 staining (Red colour) and the nuclei (Green colour) were obtained with the 595nm filter and the UV filter settings respectively.

Figure 15

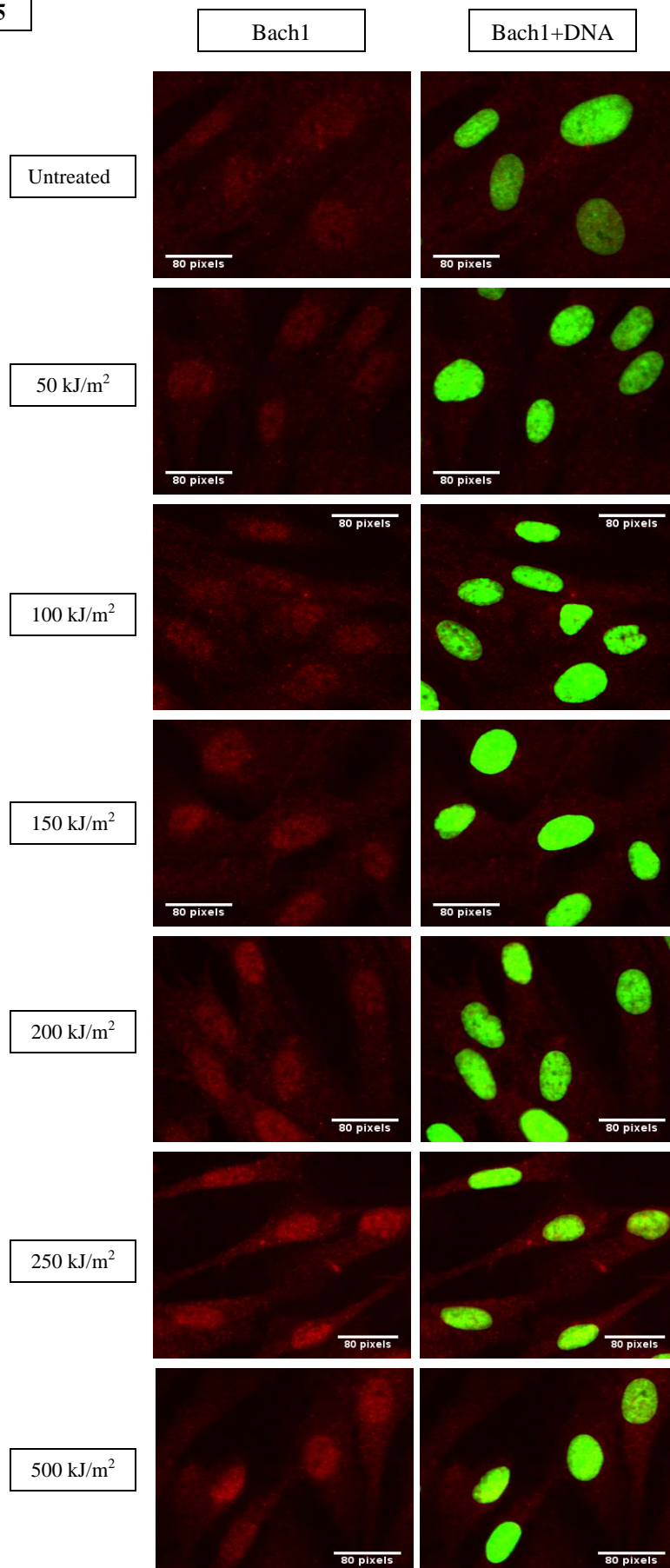


Figure 15 Effects of different doses of UVA irradiation on Bach1: FEK4 cells were irradiated with 50, 100, 150, 200, 250 and 500kJ/m² UVA doses respectively and incubated in conditioned media at 37°C for 8h. The cells were fixed at the indicated time-points with 4% PFA and immuno-staining was performed using anti-Bach1 goat polyclonal primary antibody followed by Alexa Fluor 595 rabbit anti-goat secondary antibody. The cell nuclei were stained with Hoechst stain. The images for the Bach1 staining (Red colour) and the nuclei (Green colour) were obtained with the 595nm filter and the UV filter settings respectively.

Figure 16

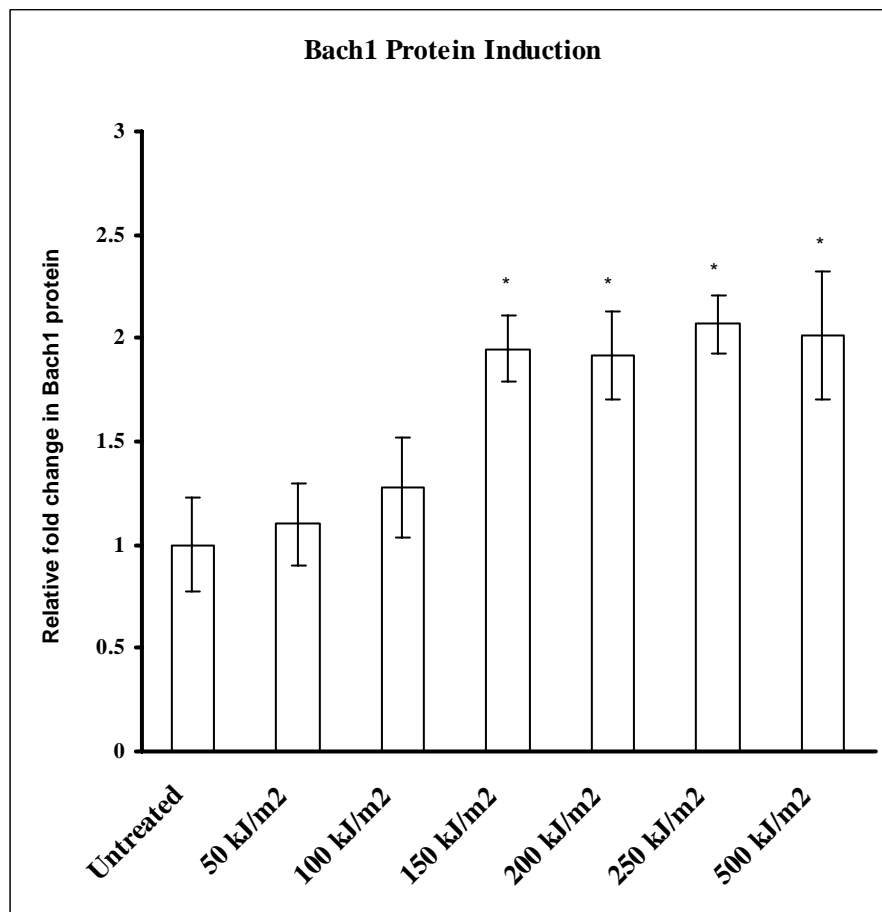


Figure 16 Effects of different doses of UVA irradiation on Bach1: Fluorescence intensity was quantified using the Image J software package. The mean gray value of 300 cells for each condition from three individual experiments was quantified for each sample and data were expressed as the relative fold increase over untreated

samples. Data are mean \pm S.D. of 3 independent experiments. The Student paired t-test was used to evaluate the statistical significance ($p < 0.05$) of each data point.

* Significance level when compared with the untreated control

Figure 17

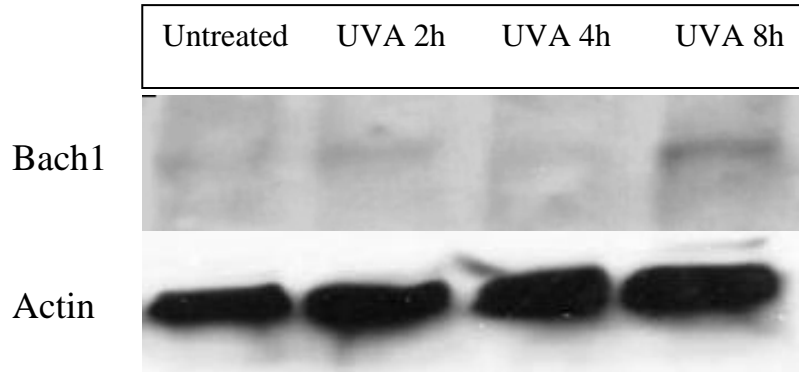


Figure 17 Bach1 protein induction following UVA irradiation: Freshly confluent FEK4 cells were irradiated with a moderate dose of UVA (250kJ/m^2) and incubated in conditioned medium at 37°C for the indicated times. Following the incubation, whole cell extracts were prepared as described in the materials and methods. For each sample, $30\mu\text{g/lane}$ of whole cell protein was loaded. Bach1 protein levels were quantified using western blot analysis. Actin protein levels were determined in all samples and used to determine equal loading. The experiment was repeated four times with identical results.

3.2 UVA irradiation promotes endogenous Bach1 protein export from the nucleus

Transcriptional regulation of the HO-1 gene involves NF-E2-Related Factor 2 (Nrf2) as a transcriptional activator and Bach-1 as a negative regulator, both of which have similar binding affinity with small Maf proteins at MARE (Maf Recognition Element) sites in the promoter of the HO-1 gene(Sun *et al.*, 2004b). Therefore the nuclear localisation of both Nrf2 and Bach1 appears to be very important in order to occupy the MARE sites. Bach1 protein carries six heme binding sites in its protein structure(Ogawa *et al.*, 2001). Binding of heme, as well as the treatment of cells with heavy metals like cadmium and arsenite promotes nuclear export of Bach1 protein(Suzuki *et al.*, 2003b;Reichard *et al.*, 2007). In view of an earlier report from our group showed that UVA irradiation causes release of heme from the microsomal heme-containing protein in the cellular environment (Kvam *et al.*, 1999a), we examined the possibility that UVA-mediated release of heme provokes Bach1 protein export. However the immediate release of heme by UVA does not seem to provoke rapid nucleus export and Bach1 protein accumulates in the nucleus up to 8 h following UVA irradiation (figure 12). In contrast to the pattern of heme release Bach1 export from the nucleus was only observed at a later time following UVA treatment. By 12 h following UVA treatment, Bach1 signal was observed predominantly in the cytoplasm in almost 90% of the cells (figure 18 and 19). A similar pattern of Bach1 protein distribution was observed up to 24 h (data not shown). Figure 18 clearly shows that although the endogenous Bach1 protein has been exported from the nucleus a high level is now observed in the cytoplasm consistence with the conclusion that UVA-mediated release of free heme in the cellular environment does not provoke Bach1 protein degradation in contrast to the situation observed after extra- cellular heme treatment (Zenke-Kawasaki *et al.*, 2007a).

Figure 18

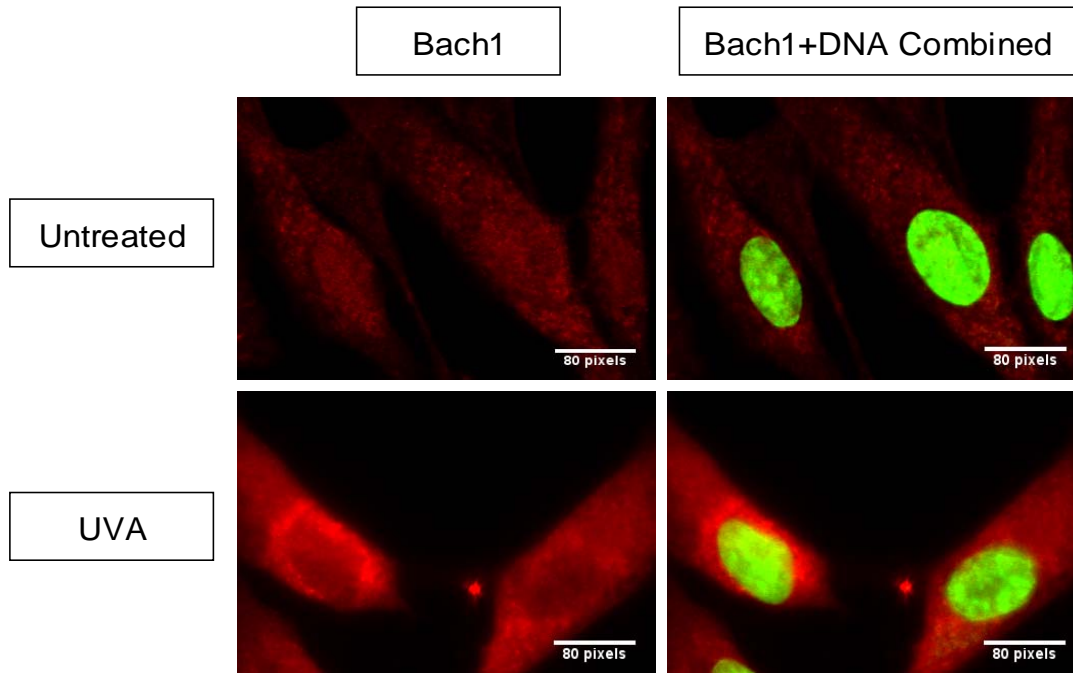


Figure 18 UVA mediated Bach1 protein export from the nucleus: FEK4 cells were UVA irradiated (250kJ/m^2) and incubated in conditioned media at 37°C for 12h. The cells were fixed with 4% PFA and immuno-staining was performed using anti-Bach1 goat polyclonal primary antibody followed by Alexa Fluor 595 rabbit anti-goat secondary antibody. The cell nuclei were stained with Hoechst stain. The images for the Bach1 staining (Red colour) and the nuclei (Green colour) were obtained with the 595nm filter and the UV filter settings respectively.

Figure 19

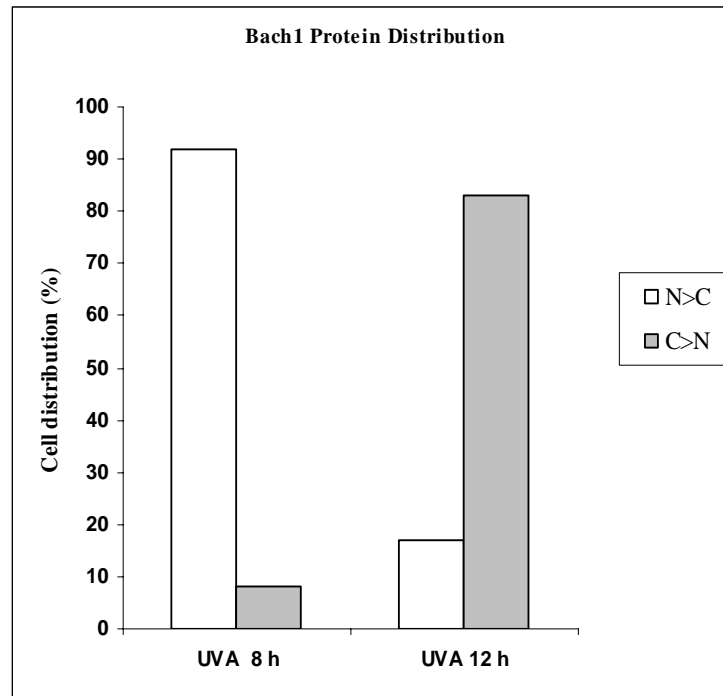


Figure 19 UVA mediated Bach1 protein export from the nucleus: Following UVA irradiation, localisation of the endogenous Bach1 protein was manually observed after 12 h incubation. Based on a predominant sub-cellular location of the endogenous Bach1 protein, cells were counted and divided in two categories N>C (Cells with predominant nuclear Bach1) and C>N (Cells with predominant cytoplasmic Bach1). The percentage distribution was calculated from 300 individual cells from three separate trials for each condition. Data are mean \pm S.D. of 3 independent experiments. The Student paired t-test was used to evaluate the statistical significance ($p < 0.05$) of each data point.

3.3 UVA released heme induces Crm1 mediated Bach1 protein export following UVA irradiation

To understand the possible mechanism underlying Bach1 protein export and to confirm involvement of UVA released heme in the export process we checked Bach1 protein localisation following UVA irradiation of heme-depleted FEK4 cells. The FEK4 cells were treated with Succinyl Acetone (SA) for 18-22 h in serum free media prior to UVA irradiation. As shown in figure 20, UVA fails to induce and export Bach1 protein from the nucleus in heme-depleted cells and the Bach1 protein stays in the nucleus 12 h following UVA treatment. We also checked the possibility that Bach1 nuclear export occurs via Crm1 protein. The Crm1 export protein was inhibited by treating cells with the Crm1 specific inhibitor Leptomycin B (LMB) prior to UVA irradiation. As for SA treatment, the LMB treatment prevented the export and the induction of Bach1 protein and the protein stayed in the nucleus. The results described in figure 20 are consistent with the conclusion that heme is involved in the Crm-1 dependant nuclear export of Bach1 protein but since there is a long delay following UVA treatment, the immediate UVA release of heme is insufficient to provoke the response.

Figure 20

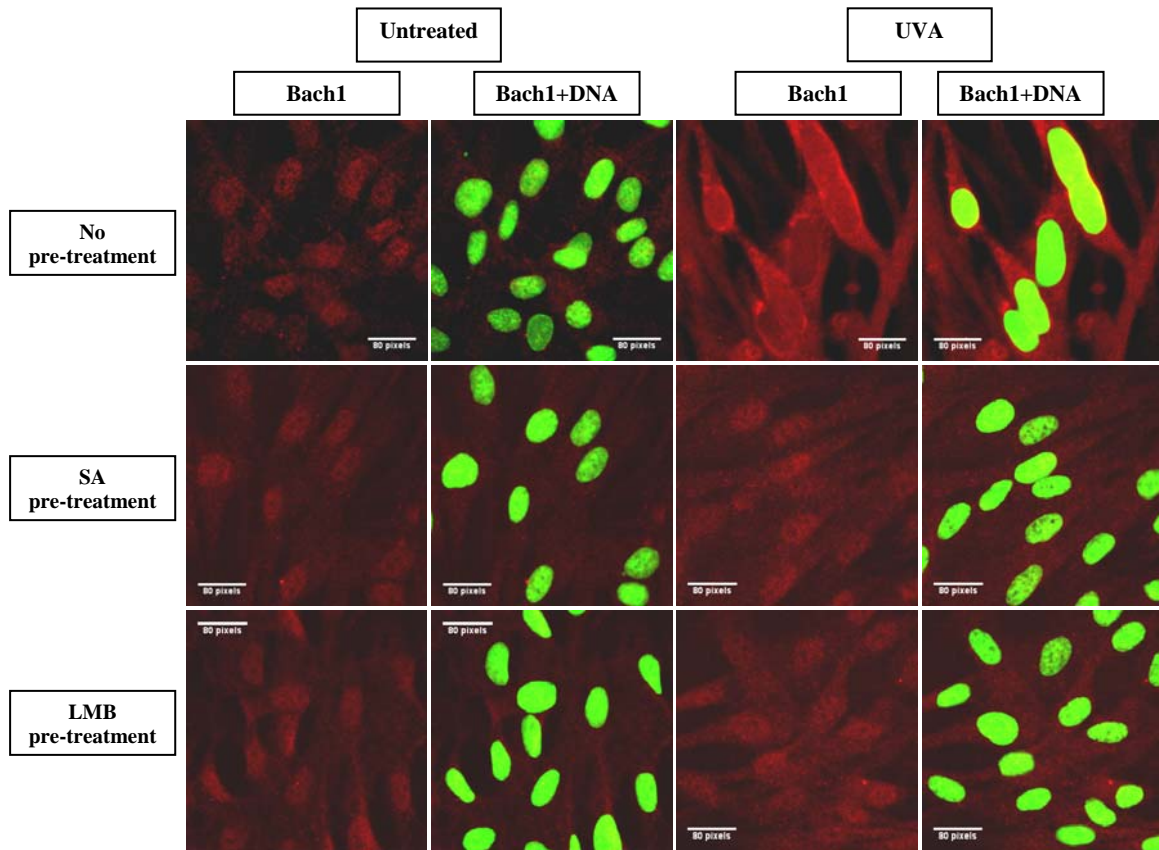


Figure 20 UVA-mediated heme release promote Bach1 protein export via Crm1 pathway: FEK4 primary human fibroblasts with no pre-treatment, SA treatment and LMB treatment were UVA irradiated (250 kJ/m^2) and incubated in conditioned media at 37°C for 12h. The cells were fixed at the indicated time-points with 4% PFA and immuno-staining was performed using anti-Bach1 goat polyclonal primary antibody followed by Alexa Fluor 595 rabbit anti-goat secondary antibody. The cell nuclei were stained with Hoechst stain. The images for the Bach1 staining (Red colour) and the nuclei (Green colour) were obtained with the 595nm filter and the UV filter settings respectively. Changes in the localisation of Bach1 protein, with and without UVA treatment were compared between the cells with no pre-treatment, SA or LMB pre-treatments.

3.4 Bach1 over-expression represses UVA-mediated HO-1 expression

To check involvement of Bach1 in UVA-mediated HO1 expression, various over-expressing constructs of Bach1 and MafK with different epitopes were prepared (Mitchell and Tyrrell unpublished data). FEK4 cells were co-transfected with constructs containing Bach1-GFP and its binding partner MafK-RFP by using Lipofectamine 2000 transfection reagent. The transfection efficiency was checked with FACS analysis, and confirmed by manual counting of the co-transfected cells by epifluorescence microscopy (results not shown). In spite of vigorous optimisation, only 15-20% of co-transfection was achieved. Since co-transfection was low, the effects of Bach1-MafK over-expression were over-shadowed by the influence of non-transfected cell populations in Western Blot analysis. To overcome this problem Immunocytochemistry was used to check the effects of Bach1 and MafK over-expression on endogenous HO-1 protein at the single cell level. In this technique, we detected the endogenous HO-1 protein by primary anti-HO-1 antibody followed by the secondary fluorescence antibody, whose peak excitation (647 nm) occurs at a different wavelength from GFP (488nm) or RFP (595 nm). Transfection with the parental vectors had no effect on UVA-modulated HO-1 expression. However, over-expression of both Bach1 and MafK represses UVA-modulated HO1 expression in individual cells so that HO-1 expression following the UVA irradiation is very low in the Bach1-MafK transfected cells compared to the HO-1 expression in the cells with parental vectors (figure 21). The change in the fluorescence intensity of HO1 staining in the cells with Bach1-MafK was quantified and expressed as relative fold change over the HO1 fluorescence intensity in the parental vector-transfected cells. Bach1-MafK transfection represses the basal HO1 expression to 70 percent of that observed in the parental vector transfected cells. In the UVA-treated cells the HO1 fold change in the Bach1-MafK co-transfected cells was 1.34 fold compared to 4.87 fold for the parental vector transfected and UVA-treated cells, a 3.5 fold reduction (figure 22).

Figure 21

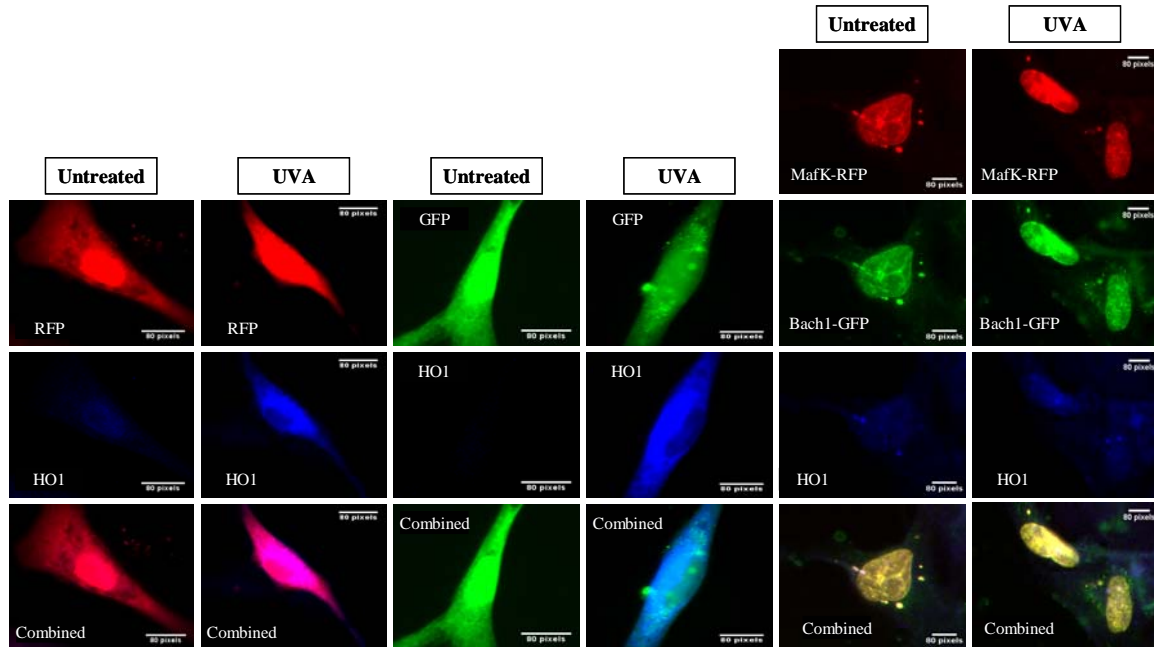


Figure 21 Bach1 over-expression represses UVA-mediated HO1 expression: The parental vectors, Bach1-GFP and MafK-RFP overexpressing constructs were transfected in FEK4 cells using Lipofectamine 2000 transfection reagents. The cells were UVA irradiated (250kJ/m²) 24 h after the transfection. The cells were fixed 12 h following UVA irradiation and immuno-staining was performed as mentioned in the material and methods. The cells were incubated with the Anti-HO1 primary antibody (1:200 dilutions) followed by the Alexa Fluor 647 SFX secondary antibody (1:1000 dilution) for 1 h. Each co-transfected cell was checked with three different filters to monitor GFP (Green colour), RFP (Red colour) and Alexa Fluor 647 (blue), and also the three filters combined. Change in the expression of HO1 protein (blue colour) in the cells transfected with Bach1 and MafK vectors with or without UVA treatment were compared with the cells transfected with just the parental vectors.

Figure 22

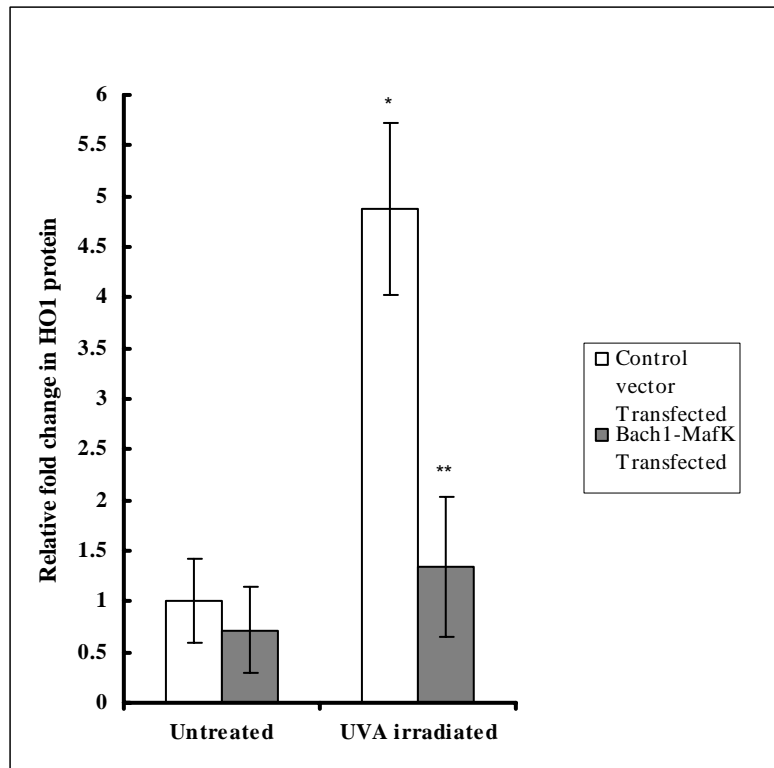


Figure 22 Bach1 over-expression represses UVA-mediated HO1 expression: Changes in the expression of HO-1 protein in the cells transfected with the parental vectors, Bach1 and MafK vectors with or without UVA treatment were quantified by the Image J software package. The change in HO-1 expression was measured as a mean gray value and presented as a mean of 300 cells from three individual trials for each sample and data were expressed as the relative fold increase over parental vector transfected untreated samples. Data are mean \pm S.D. of 5 independent experiments. The Student paired t-test was used to evaluate the statistical significance ($p < 0.05$) of each data point.

* Significance level when compared with the untreated control

** Significance level when compared with the UVA irradiated control vector

3.5 Silencing of Bach1 induces HO-1 protein expression

The role of Bach1 in UVA mediated HO-1 protein induction was further examined using Bach1 gene specific siRNAs. Bach1 expression was silenced by using chemically synthesized siRNAs targeting three different regions of the Bach1 gene (*). FEK4 cells were transfected with 5nM and 30 nM concentrations of Bach1 specific siRNAs for 48h and 72h. Bach1 and HO-1 protein levels were observed by Western blot analysis. Non-targeted siRNAs (Scrambled siRNA) were transfected to confirm specificity of the Bach1 siRNAs. As shown in figure 23A, 30nM concentrations of Bach1 specific siRNA reduces Bach1 protein expression several-fold demonstrating that the siRNA's are functional. A strong increase in the constitutive expression of HO-1 protein was observed in cells in which the Bach1 gene had been silenced, compared to cells transfected with non-specific siRNA (figure 23A). Cells irradiated with UVA have a higher induction of HO-1 when transfected with 30nM Bach1 specific siRNAs in contrast to cells transfected with scrambled siRNA and vehicle (Optimem media only) (figure 23B). Similar results to the above were obtained by using a Bach1 specific siRNA cocktail generated by targeting a 176bp region of the Bach1 gene's open reading frame by using individual components (figure 24). Cells were transfected with 5nM of the Bach1 specific siRNA cocktail for 48h. Basal and UVA-induced HO-1 protein levels were significantly higher in the cells with the Bach1 specific siRNA cocktail compared to the cells without siRNAs. Taken together, these data provide strong evidence for the direct involvement of Bach1 in UVA-mediated HO-1 expression.

*** This experiment was performed by Dr. Julia Zhong**

Figure 23

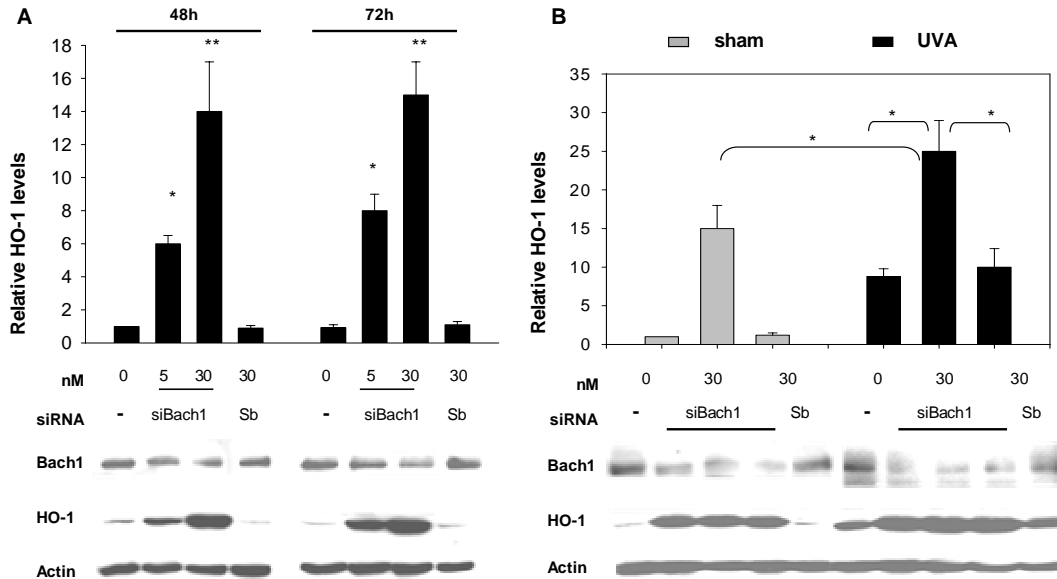


Figure 23 Silencing of Bach1 induces basal and UVA-induced HO-1 expression: FEK4 cells were transfected with the indicated amounts of Bach1 specific siRNA or non-specific scrambled siRNAs. Following transfection, the cells were incubated for 48h or 72h time intervals in conditioned media at 37°C(A). After 48 h of transfection FEK4 cells were treated with UVA (250kJ/m²) and incubated in conditioned media at 37°C for 8 h.

* Significance level when compared with the non-specific scrambled siRNA control

** Significance level when compared with the 5nM Bach1 siRNA sample

(B). Total cellular protein was isolated as mentioned in the materials and methods. Levels of Bach1 and HO-1 proteins in the samples were quantified using Western blot analysis. Actin levels were determined in all samples and used for normalizing Bach1 and HO1 protein levels.

* Significance level when compared with the non-specific scrambled siRNA and the untreated sample.

Figure 24

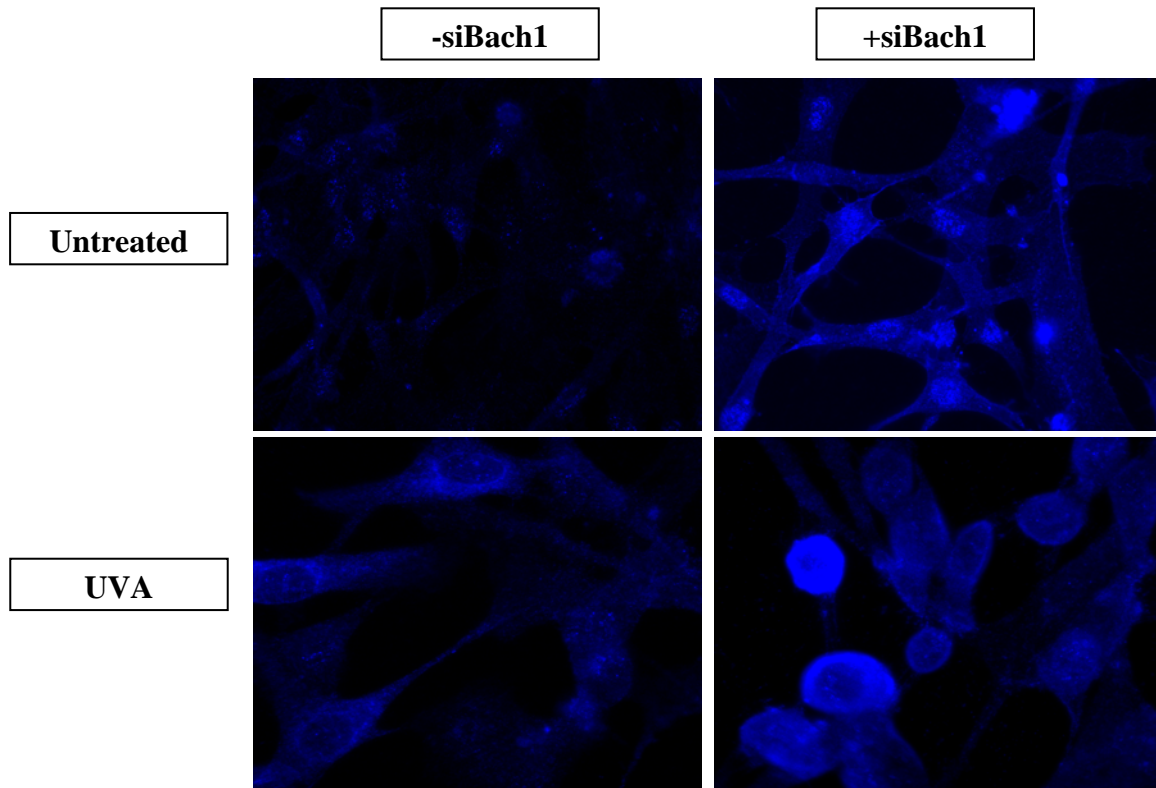


Figure 24 Silencing of Bach1 induces basal and UVA-induced HO-1 expression: FEK4 cells were transfected for 4-6h with 5nMolar of Bach1 specific siRNA cocktail by using Lipofectamine 2000 reagent. Following treatment cells were further incubated for 48 h in conditioned media at 37°C. Following the incubation FEK4 cells were irradiated with a moderate dose of UVA (250kJ/m²) and incubated in conditioned media at 37°C for 12 h. The cells were fixed with 4% PFA and immunostaining was performed using anti-HO-1 mouse monoclonal primary antibody followed by Alexa Fluor 647 goat anti-mouse secondary antibody. The images for the HO-1 staining (Blue colour) were obtained with the 647nm filter settings. Changes in the levels of HO-1 proteins with and without siBach1 were compared before and after UVA irradiation.

3.6 Refractoriness of HO-1 protein

A previous report from this laboratory has demonstrated development of an acquired refractoriness (resistance) of the heme oxygenase-1 gene to induction by a second dose of UVA irradiation (Noel and Tyrrell, 1997b). The study demonstrated that complete refractoriness to re-induction of the HO-1 gene occurs when a challenge dose was given 48h after a first dose while a challenge dose after 15h and 72h incubation resulted in partial refractoriness. Interestingly other potent HO-1 inducers; Na-arsenite and hydrogen peroxide do not develop refractoriness and the conclusion from the study was that the refractoriness to HO-1 induction is an exclusive response to UVA irradiation and heme treatment. In order to understand the mechanism of the refractoriness response we have focussed on what may occur when a challenge dose is given 48 h after the first dose. In the original study, measurement was restricted to estimation of mRNA accumulation and so we expanded this to investigate HO-1 protein levels following re-irradiation. We observed that re-irradiation 48h after a first UVA dose leads to significantly lower levels of HO-1 protein compared with the control samples in which no pre-irradiation was given (figure 25) entirely consistent with previous observation on mRNA accumulation.

Figure 25

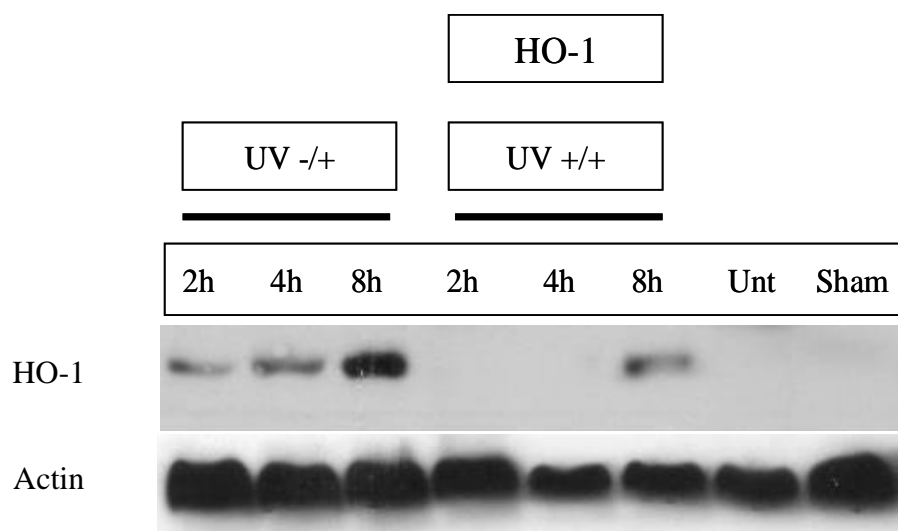


Figure 25 Refractoriness of HO-1 protein following a second UVA dose: FEK4 cells were cultured in 10 cm tissue culture dishes with two cover slips in each plate. One set of plates were pre-irradiated with UVA (250kJ/m²) and reincubated in conditioned medium for 48 h at 37°C. Following the incubation, both sets of plates were treated with a second dose of UVA (250kJ/m²) followed by further incubation. Following incubation, whole cell extracts were prepared as described in the materials and methods. For each sample, 30µg/lane of whole cell protein was loaded. HO-1 protein levels were quantified using Western blot analysis. Actin protein levels were determined in all samples and used to determine equal loading. HO-1 levels in the samples treated with a single UVA dose (UVA -/+) were compared with doubly irradiated samples (UVA +/+), untreated sample (Unt) and Sham treated sample. The Sham sample was treated exactly the same as the UVA treated samples, but was not irradiated. The experiment was repeated four times with identical results.

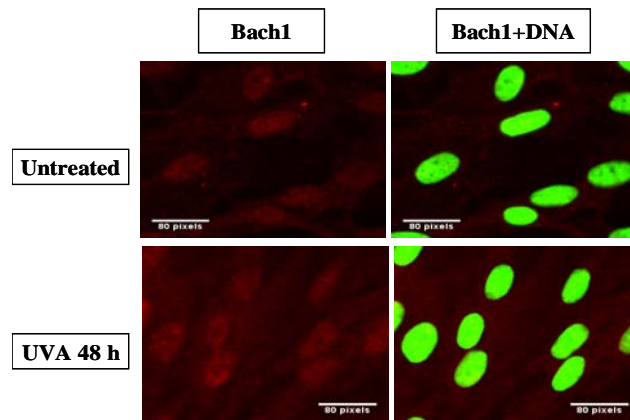
3.7 A role of Bach1 in HO-1 refractoriness (48 h)

Heme appears to play a central role in regulation of the HO-1 gene by influencing the cellular localisation and the expression levels of both the transcription factors, Bach1 and Nrf2 (Sun *et al.*, 2004a). Interestingly, loss of total heme-containing proteins via UVA-mediated degradation and as a result of degradation by increased level of HO-1 following UVA treatment do not recover within 48 h and upon the challenge dose free heme release was not observed (Kvam *et al.*, 1999b). Therefore it was hypothesised that in the cellular environment where UVA-released heme had been degraded by increased levels of HO1 protein, the expression and localisation of Nrf2 and Bach1 may play a central role in the development of refractoriness of the HO-1 gene to induction by a second UVA dose. We tested this hypothesis by monitoring protein levels and localisation of Nrf2 and Bach1 following a second UVA dose and compared these results with those obtained after a single dose.

To investigate a role of Bach1 protein in the mechanism of refractoriness we first studied Bach1 protein expression following UVA irradiation for up to 48h. As mentioned in Section 3.1 and 3.3, we observed induction and accumulation of the Bach1 protein by 8h incubation following UVA irradiation and Bach1 protein is then exported from the nucleus by 12 h. Interestingly no decrease in the level of the exported Bach1 protein was observed and the elevated levels of Bach1 protein were observed in the cytoplasm until 24 h (data not shown). Interestingly, Bach1 protein signals were observed in the nucleus prior to the challenge dose at 48h (figure 26) indicating that Bach1 protein although initially exported to the cytoplasm returns to the nucleus in between 24 to 48 h and the level of Bach1 in the nucleus at this later time remains high compared with the untreated cells (figure 26). Further UVA irradiation of cells 48 h after pre-irradiation caused further induction and accumulation of Bach1 protein in the nucleus up to at least 8h (figure 27). The change in the fluorescence intensity of the Bach1 staining in the cells was quantified and expressed as relative fold change over the Bach1 fluorescence intensity in the untreated cells (figure 28). The results were further confirmed by the checking Bach1 protein level in the whole cell protein lysates. As shown in figure 29 Bach1 protein

increases following UVA irradiation and there is a further increase following the second dose.

Figure 26



Bach1 protein levels 48h following UVA irradiation: FEK4 cells were irradiated with a moderate dose of UVA(250kJ/m²). Following the treatment the cells were incubated in conditioned media for 48h. Bach1 protein levels (Red) in the cells with UVA treatment were detected and compared with Bach1 protein levels in untreated cells as mentioned in the materials and methods.

Figure 27

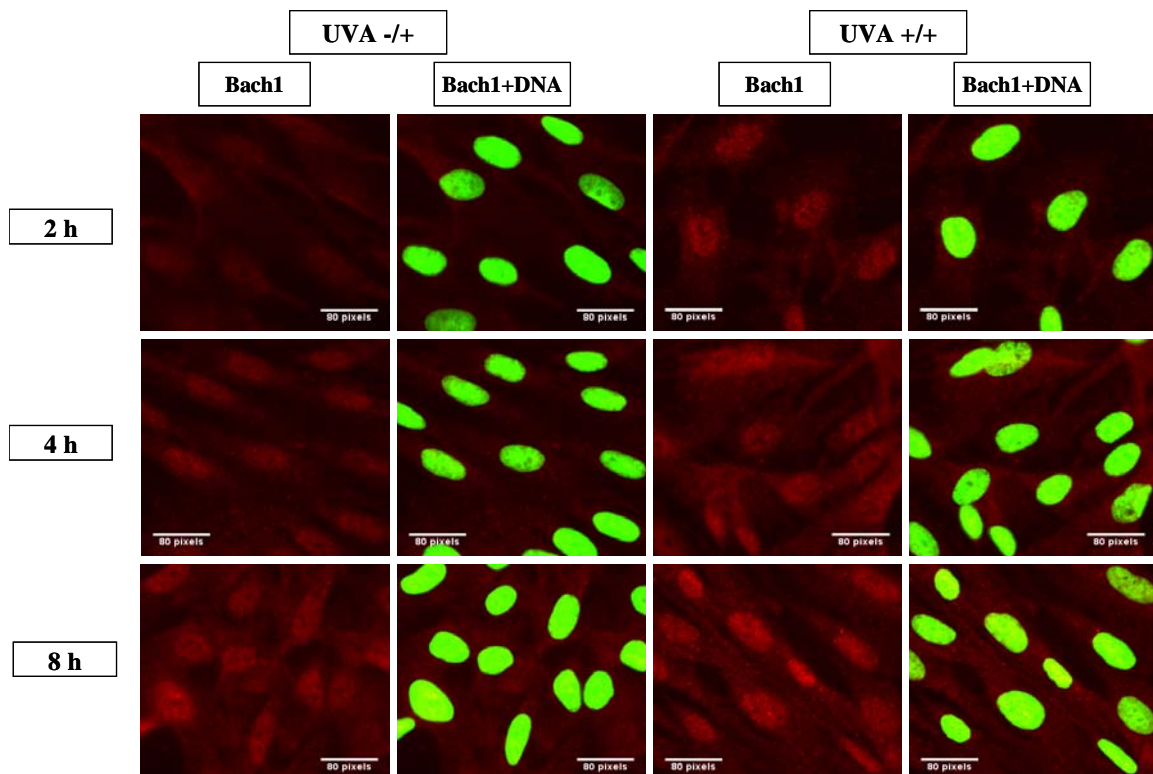


Figure 27 Bach1 protein stays in the nucleus following the challenge dose: FEK4 cells were cultured in 10 cm tissue culture dishes with two cover slips in each plate. Half of the plates were preirradiated with UVA (250kJ/m²) and reincubated in conditioned medium for 48 h at 37°C. Following the incubation, both sets of plates were irradiated with a challenge dose similar to the first dose followed by a further incubation period. For each condition, levels of Bach1 protein was identified by Immunocytochemistry (ICC) analysis as described in the material and methods. Levels of Bach1 proteins in the samples given a single UVA dose (UVA -/+) were compared with the doubly-irradiated samples (UVA +/+).

Figure 28

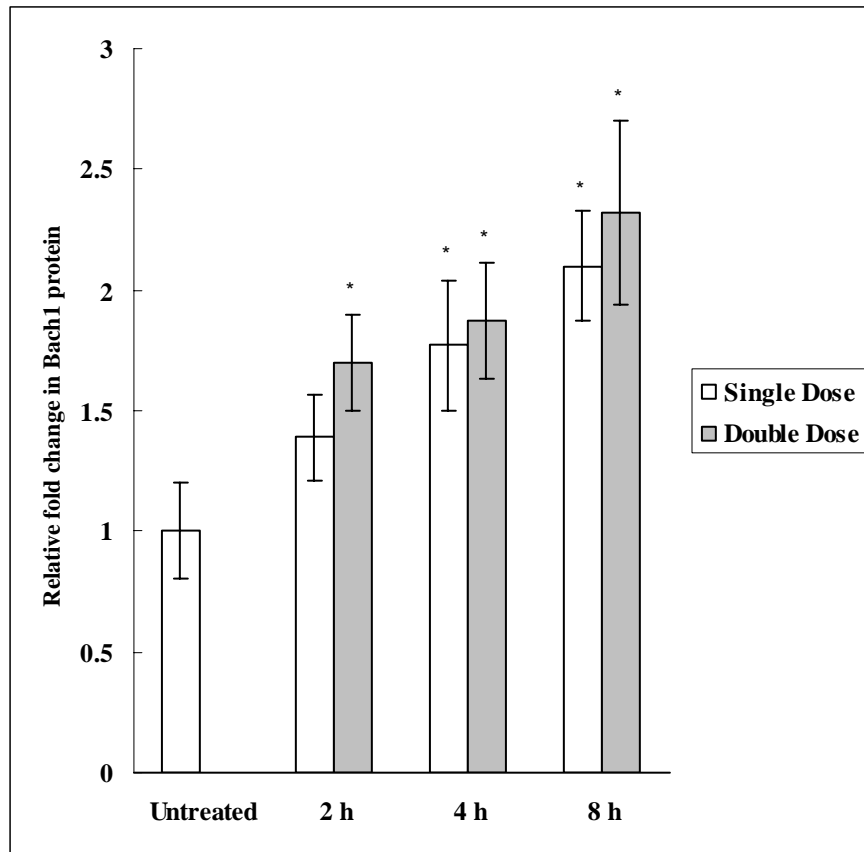


Figure 28 Higher Bach1 protein levels in the nucleus following the challenge dose: The fluorescence intensity was quantified using the Image J software package. The mean gray value of 300 cells for each condition from three individual experiments was quantified for each sample and data were expressed as the relative fold increase over untreated samples. Data are the mean \pm S.D. of 4 independent experiments.

The Student paired t-test was used to evaluate the statistical significance ($p < 0.05$) of each data point.

* Significance level when compared with the untreated control

Figure 29

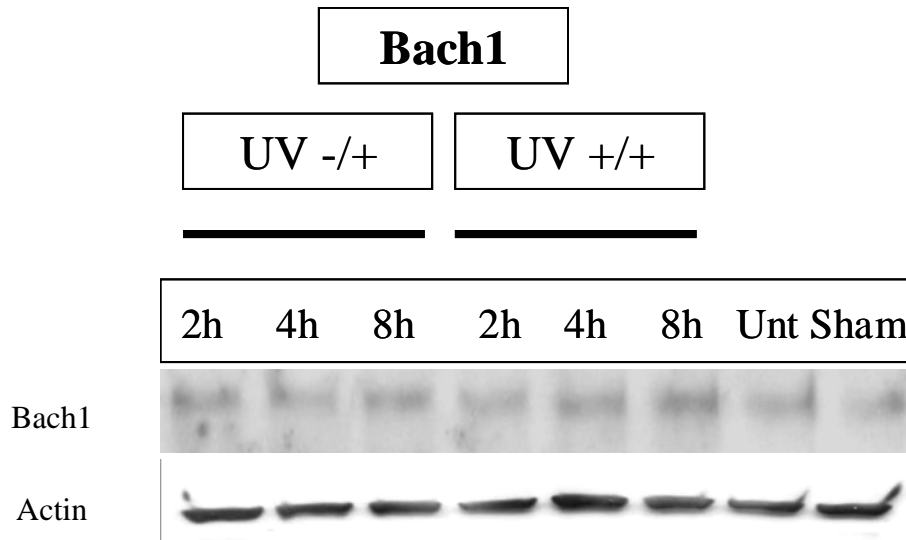


Figure 29 High Bach1 protein levels following the challenge dose: For each sample, 30µg/lane of whole cell protein was loaded. Bach1 protein levels were quantified using Western blot analysis. Actin protein levels were determined in all samples and used for normalizing Bach1 protein levels. Bach1 levels in the samples treated with single UVA dose (UVA -/+) were compared with those in the doubly irradiated samples (UVA +/+), untreated sample (Unt) and sham treated sample. The Sham sample was treated exactly the same as the UVA treated samples, but was not irradiated.

3.8 A role of Nrf2 in HO1 refractoriness (48 h)

Nrf2 is a potent transcriptional activator of the HO-1 gene. It has also been reported that under homeostatic conditions Nrf2 protein binds to the cytoplasmic protein Keap1 which promotes ubiquitination in order to maintain a low level of Nrf2 in the cytoplasm. Results from this laboratory indicate that UVA irradiation promotes immediate nuclear accumulation of Nrf2 protein in primary human skin fibroblasts (Edwards and Tyrrell unpublished observations). Nrf2 activity is regulated by heme which increases the stability of the short-lived protein Nrf2 (Alam *et al.*, 1999; Alam *et al.*, 2003). Interestingly, following the activation of target genes, the cytoplasmic protein, Keap1, enters in to the cell nucleus and promotes the degradation of Nrf2 protein (Nguyen *et al.*, 2003). Based on such results it was hypothesised that in the cellular environment where UVA-induced Nrf2 has been degraded by Keap1-mediated ubiquitination mechanism within 48 h and the lower levels of heme-containing proteins in the cells, Nrf2 may not accumulate in the nucleus following a second UVA dose which may lead to refractoriness of HO-1 gene. We tested this hypothesis by measuring Nrf2 protein levels and sub-cellular localisation at various time intervals following a second dose of UVA radiation. As shown in figure 31, UVA irradiation resulted in nuclear accumulation and activation of the Nrf2 protein. Interestingly, low levels of Nrf2 protein were observed in the cell cytoplasm 48h after the first dose indicating that levels of Nrf2 protein, which had been induced and then accumulated in the nucleus, returned back to the low basal level within two days (figure 30). A significantly lower level of the protein was detected in the nucleus after UVA irradiation of pre-irradiated cells when compared to cells that had not been pre-irradiated (figure 31 and 32). The results were confirmed by checking Nrf2 protein levels in the whole cell protein extracts (figure 33). These results are consistent with the concept that a lack of Nrf2 protein accumulation in the cell nucleus following a second UVA dose contributes to the development of refractoriness to re-induction of the HO-1.

Figure 30

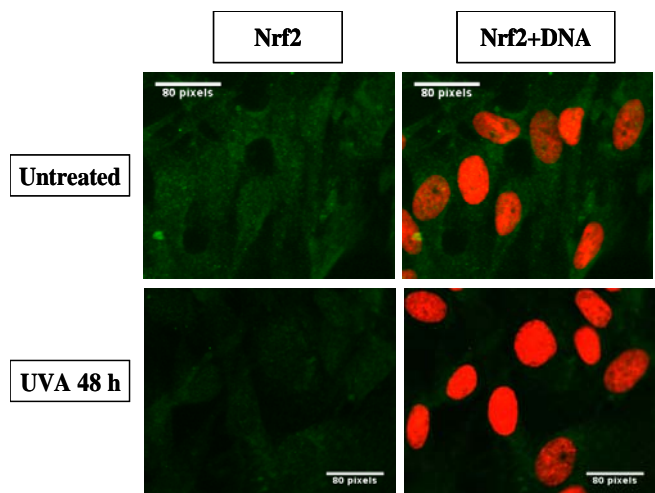


Figure 30 Lower Nrf2 protein levels 48h following UVA irradiation: Freshly confluent FEK4 cells were irradiated with a moderate dose of UVA. Following the treatment the cells were incubated in conditioned media for 48h. Nf2 protein levels in the cells with UVA treatment were detected and compared with Nrf2 protein levels in untreated cells as mentioned in the materials and methods.

Figure 31

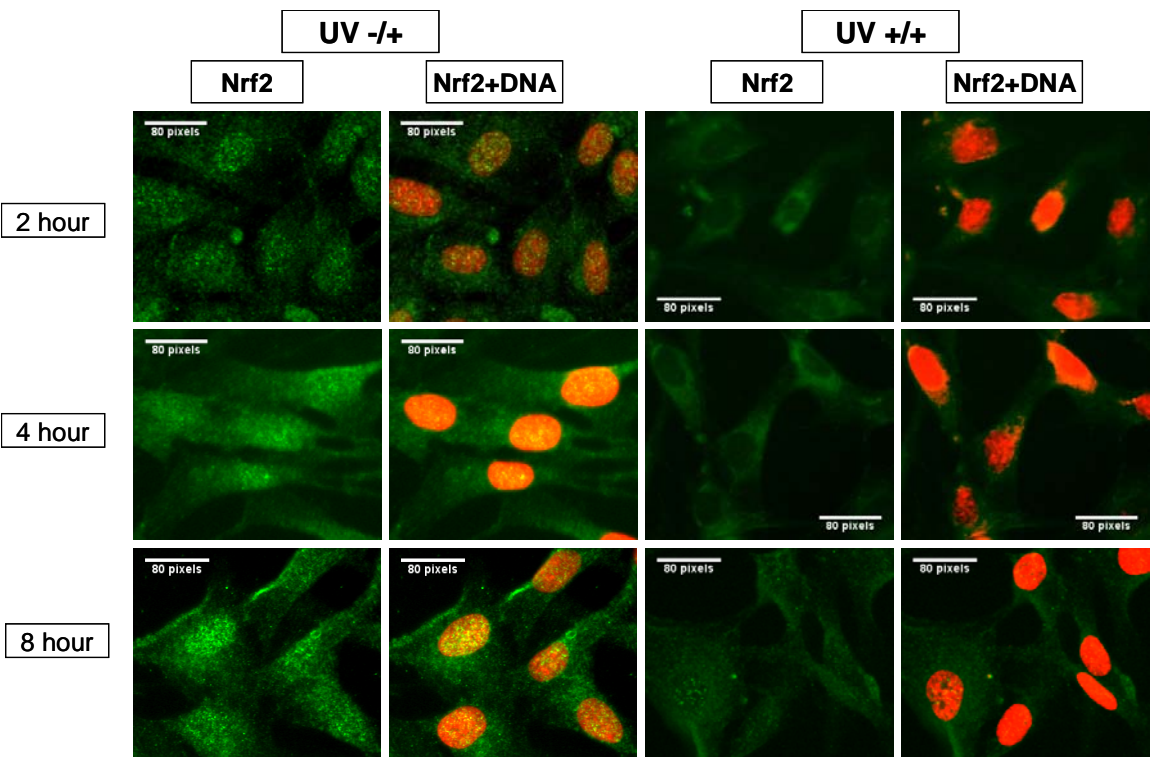


Figure 31 Nrf2 fails to induce following a second UVA dose: FEK4 cells were UVA-preirradiated (250kJ/m²) and incubated for 48 h at 37°C in conditioned media before a second irradiation under similar conditions. For each condition the coverslips were collected and Immuno-staining was performed as described in the materials and methods. Levels of Nrf2 protein in the control samples with single UVA dose (UVA -/+) were compared with doubly irradiated samples (UVA +/+).

Figure 32

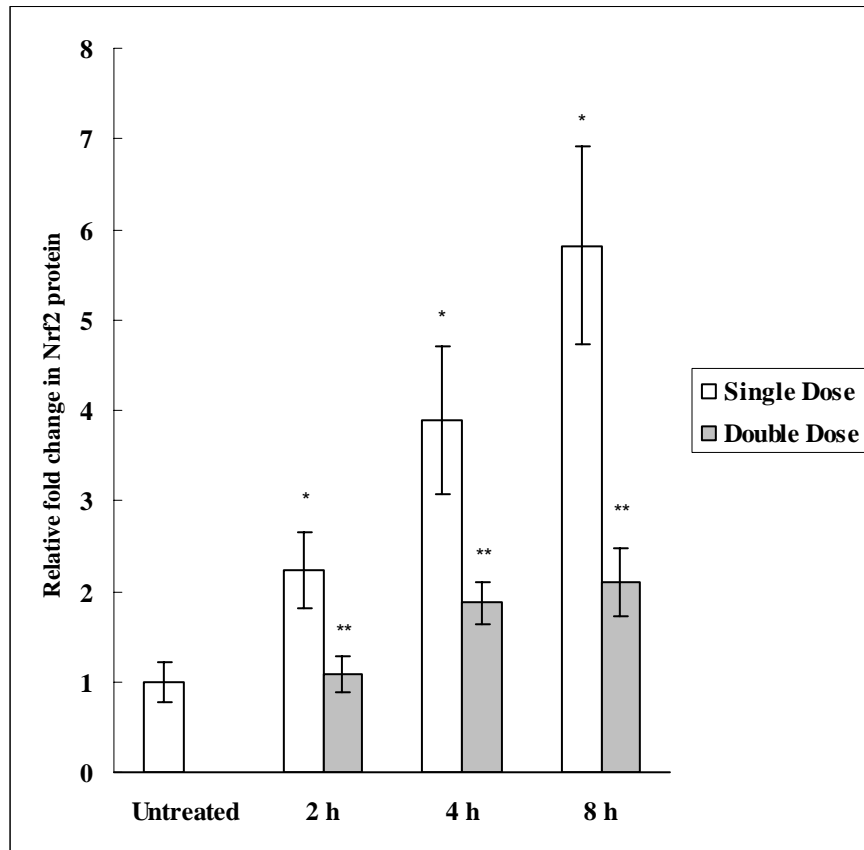


Figure 32 Nrf2 fails to induce following a second UVA dose: The Image J software package was used to quantify fluorescence intensity for each sample. The graph shows the fold-change in Nrf2 levels over untreated samples for each condition. The Student paired t-test was used to evaluate the statistical significance (p<0.05) of each data point.

*** Significance level when compared with the untreated control**

**** Significance level when compared with the samples with a single UVA dose**

Figure 33

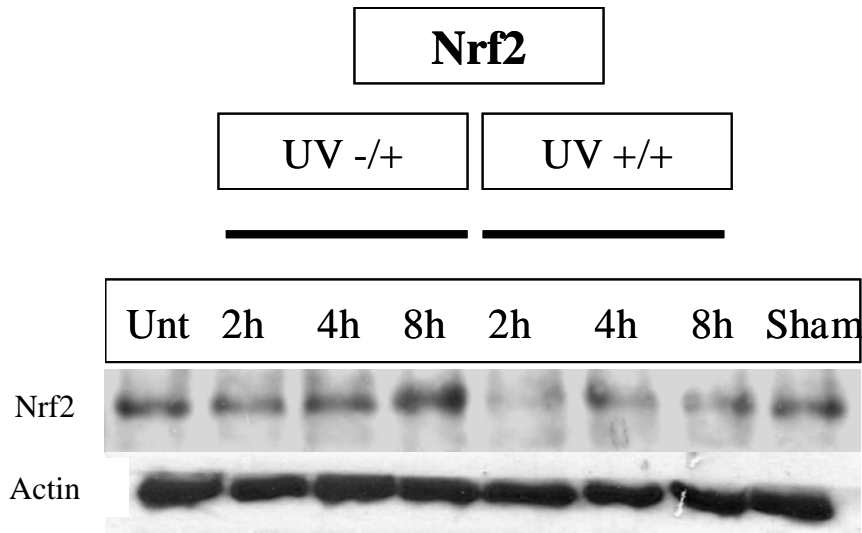


Figure 33 Nrf2 fails to induce following a second UVA dose: FEK4 cells were UVA-preirradiated (250kJ/m^2) and incubated for 48 h at 37°C in conditioned media before a second irradiation under similar conditions. Nrf2 protein levels were quantified using Western blot analysis. Actin protein levels were determined in all samples and used for normalizing Nrf2 protein levels. Nrf2 levels in the samples treated with single UVA dose (UVA -/+) were compared with those in the doubly irradiated samples (UVA +/+), untreated sample (Unt) and Sham treated sample. The Sham sample was treated exactly the same as the UVA treated samples, but was not irradiated. This experiment was repeated 4 times with identical results.

3.9 The mechanism of HO1 refractoriness (72 h)

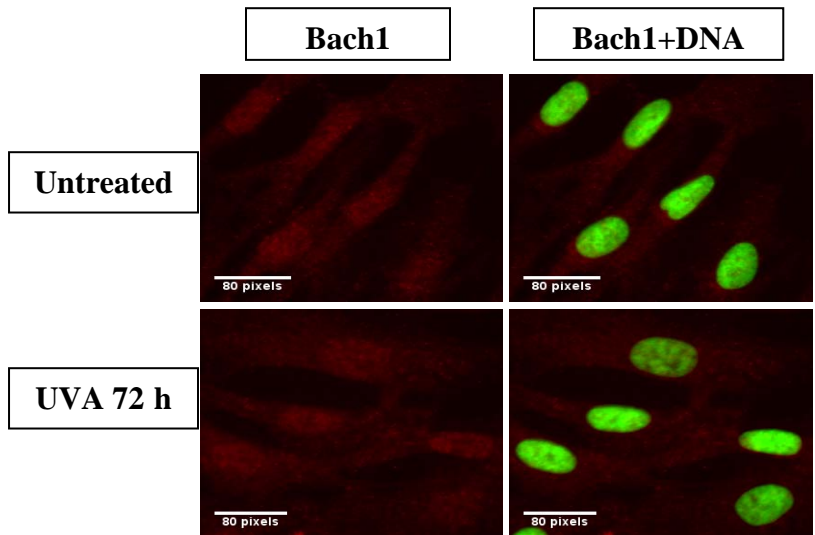
A partial return of responsiveness of the HO-1 gene to re-induction by UVA irradiation was observed after a 72 h time interval (Noel and Tyrrell, 1997a). The mechanism was investigated by monitoring Nrf2 and Bach1 protein levels by Immunocytochemistry and western blot analysis following a challenge dose at various time points.

FEK4 cells were grown in the 10cm plates with two coverslips in each plate. One set of plates were pre-treated with UVA radiation (250kJ/m^2) and incubated in conditioned media for 72 h. Following the incubation both sets of plates were treated with UVA irradiation and incubated for the indicated times. As mentioned in figure 34 the Bach1 protein level was checked prior to the challenge dose. Interestingly Bach1 protein level was similar to the untreated cells and Bach1 protein signals were observed in the nucleus prior to the challenge dose indicating that Bach1 protein once induced following UVA irradiation returns to the basal level within 72h (figure 34). Further UVA irradiation of cells 72 h after pre-irradiation caused further induction and accumulation of Bach1 protein in the nucleus up to at least 8h (figure 35). The change in the fluorescence intensity of the Bach1 staining in the cells was quantified and expressed as relative fold change over the Bach1 fluorescence intensity in the untreated cells (Figure 36). The results were further confirmed by checking the Bach1 protein level in the whole cell protein lysates. As shown in figure 37, Bach1 protein levels increase following UVA irradiation and further increase following a second dose.

Nrf2 protein levels were also checked in cells 72h following UVA irradiation. Interestingly, a low level of Nrf2 protein was observed in the cell cytoplasm prior to a second dose indicating that Nrf2 protein which had been induced and accumulated in the nucleus following UVA irradiation, returned back to basal low levels within 72h (figure 38). A significantly lower level of the protein was detected in the nucleus after UVA irradiation of pre-irradiated cells when compared to cells that had not been pre-irradiated (figure 39, 40 and 41). Interestingly, faint Nrf2 protein signals were observed in the nucleus following the challenge dose. The results were confirmed by checking Nrf2

protein levels in the whole cell protein extracts (figure 39). These results are consistent with the concept that the lower level of Nrf2 protein observed in the cell nucleus following a second UVA dose contributes to the partial development of refractoriness to re-induction of the HO-1 gene.

Figure 34



Bach1 protein levels 72h following UVA irradiation: FEK4 cells were irradiated with a moderate dose of UVA. Following the treatment the cells were incubated in conditioned media for 72h. Bach1 protein levels in the cells with UVA treatment were detected and compared with Bach1 protein levels in untreated cells.

Figure 35

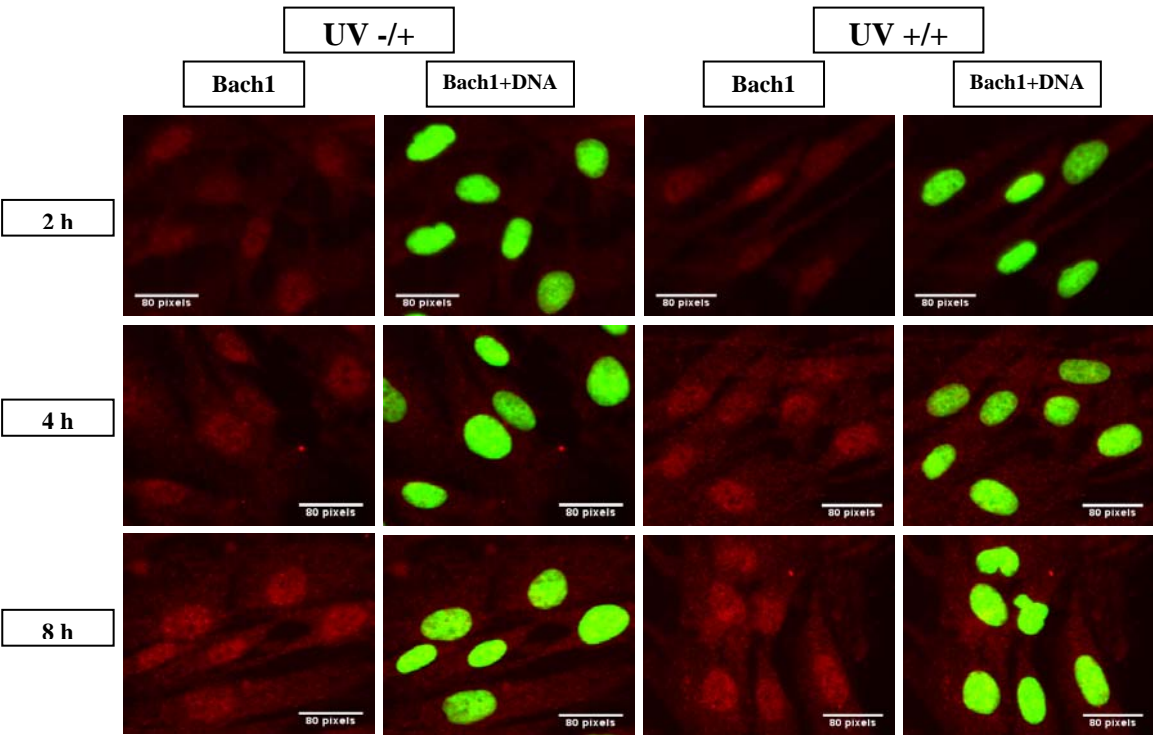


Figure 35 Nuclear accumulation of Bach1 following a second UVA dose: Bach1 protein FEK4 cells were cultured in 10 cm tissue culture dishes with two cover slips in each plate. Half of the plates were preirradiated with UVA (250kJ/m²) and reincubated in conditioned medium for 72 h at 37°C. Following the incubation, both sets of plates were irradiated with a challenge dose similar to the first dose followed by a further incubation period. For each condition, levels of Bach1 protein was identified by Immunocytochemistry (ICC) analysis as described in the material and methods. Levels of Bach1 proteins in the samples given a single UVA dose (UVA -/+) were compared with the doubly-irradiated samples (UVA +/+).

Figure 36

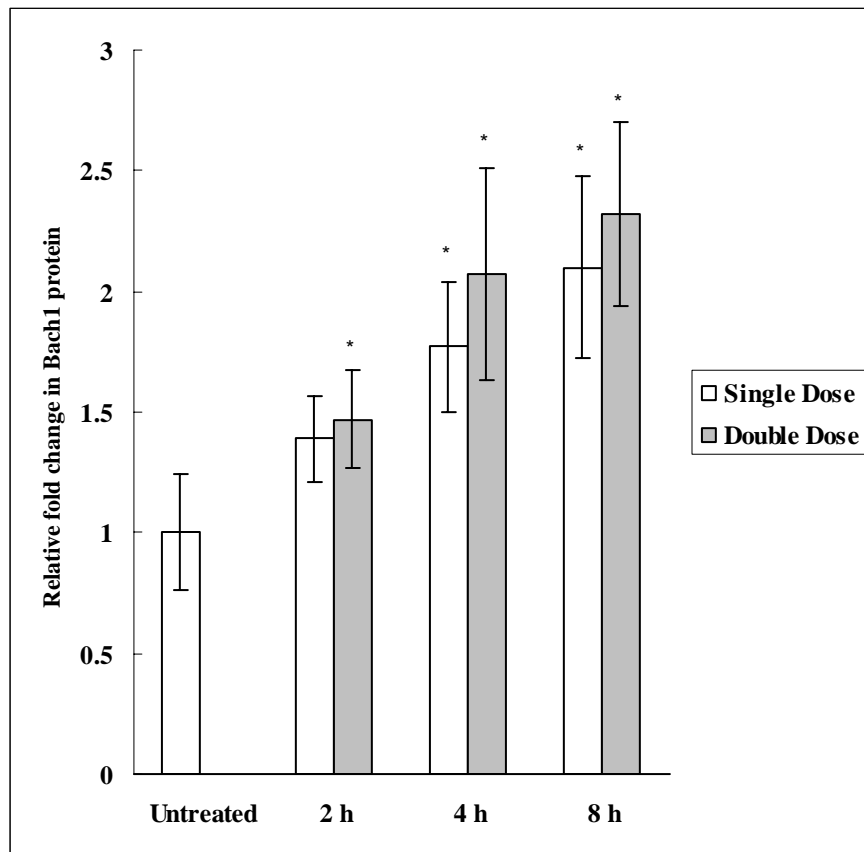


Figure 36 The fluorescence intensity was quantified using the Image J software package. The mean gray value of 300 cells for each condition from three individual experiments was quantified for each sample and data were expressed as the relative

fold increase over untreated samples. Data are the mean \pm S.D. of 4 independent experiments. The Student paired t-test was used to evaluate the statistical significance ($p < 0.05$) of each data point.

* Significance level when compared with the untreated control

Figure 37

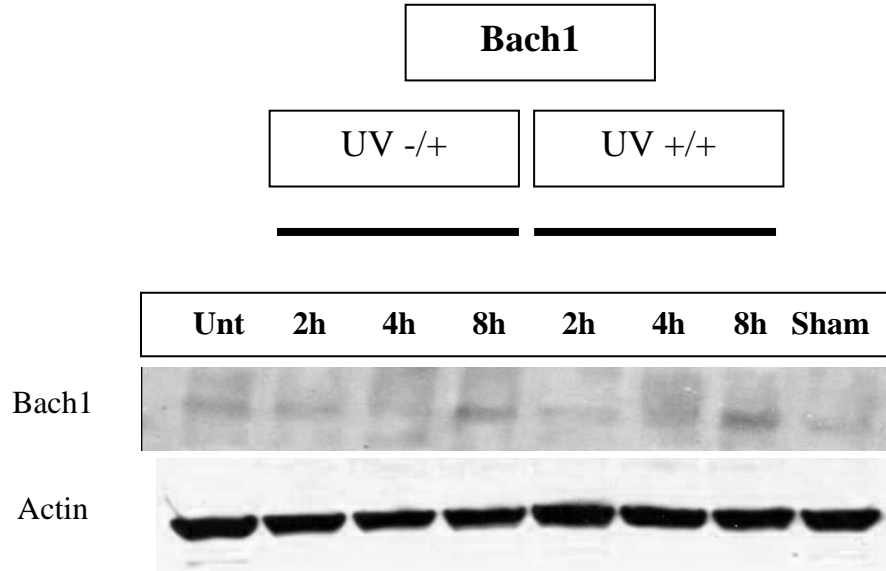
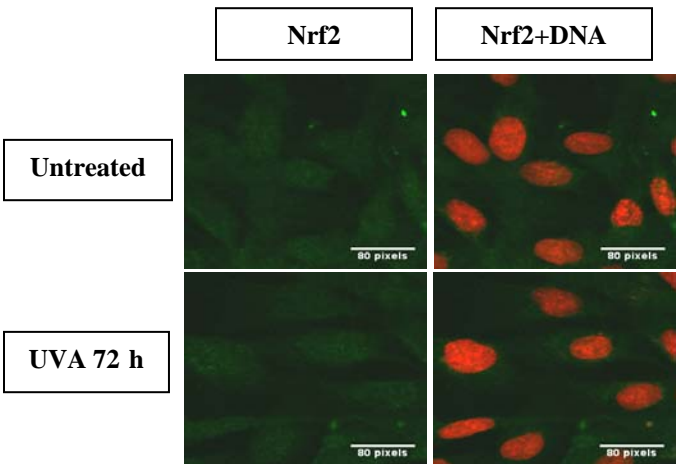


Figure 37 Bach1 protein induction following a second dose: For each sample, 30 μ g/lane of whole cell protein was loaded. Bach1 protein levels were quantified using Western blot analysis. Actin protein levels were determined in all samples and used for normalizing Bach1 protein levels. Bach1 levels in the samples treated with single UVA dose (UVA -/+) were compared with those in the doubly irradiated samples (UVA +/+), untreated sample (Unt) and sham treated sample. The Sham sample was treated exactly the same as the UVA treated samples, but was not irradiated.

Figure 38



Nrf2 protein levels come back to basal lower level within 72h: Freshly confluent FEK4 cells were irradiated with a moderate dose of UVA(250kJ/m²). Following the treatment the cells were incubated in conditioned media for 72h. Nf2 protein levels (green colour) in the cells with UVA treatment were detected and compared with Nrf2 protein levels in untreated cells.

Figure 39

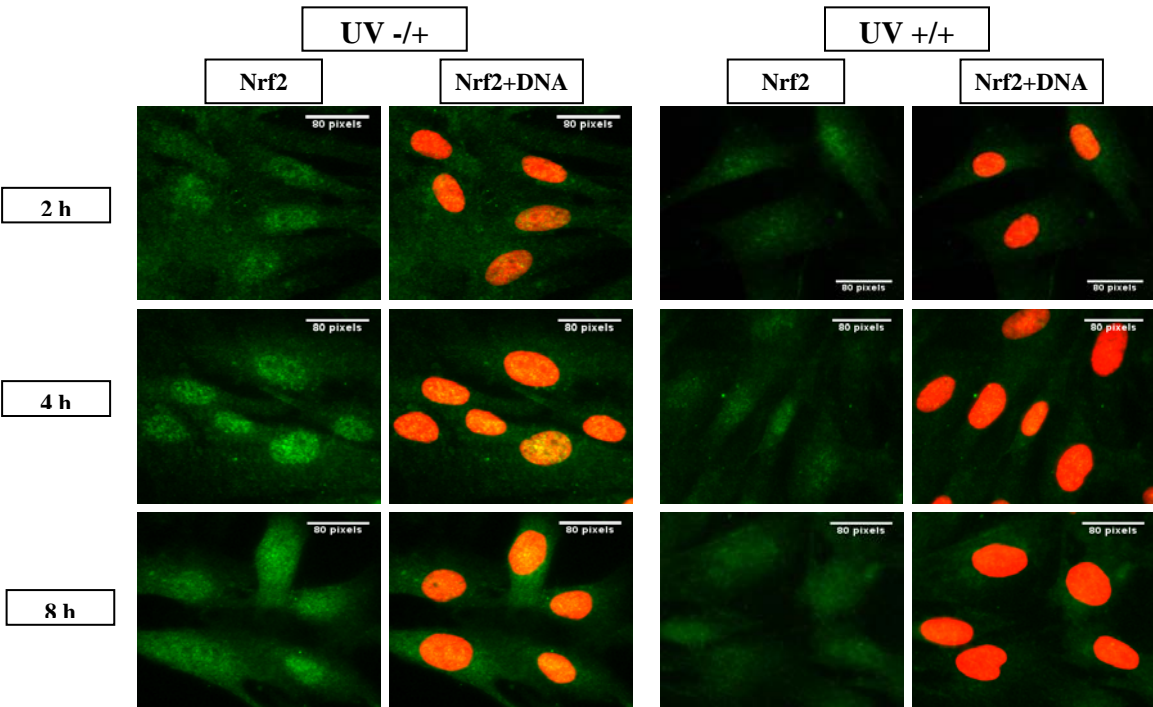


Figure 39 No Nrf2 protein induction following a second dose: FEK4 cells were UVA-preirradiated (250kJ/m²) and incubated for 72 h at 37°C in conditioned media before a second irradiation under similar conditions. For each condition the coverslips were collected and Immuno-staining was performed as described in the materials and methods. Levels of Nrf2 protein in the control samples with single UVA dose (UVA -/+) were compared with doubly irradiated samples (UVA +/+).

Figure 40

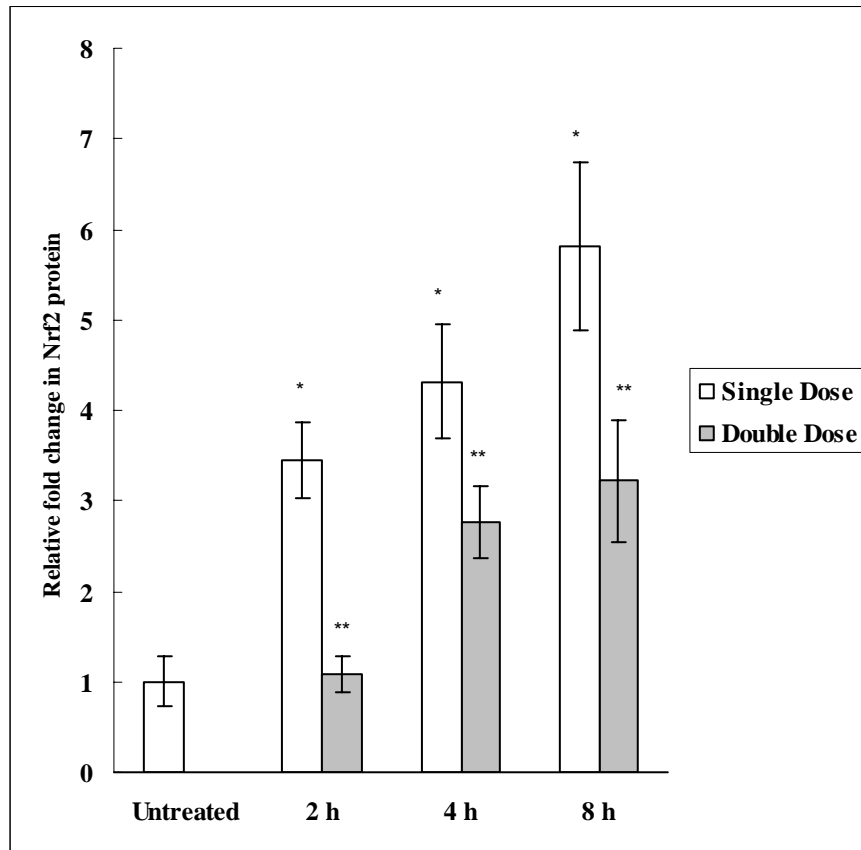


Figure 40 The Image J software package was used to quantify fluorescence intensity for each sample. The graph shows the fold-change in Nrf2 levels over untreated samples for each condition. The Student paired t-test was used to evaluate the statistical significance ($p < 0.05$) of each data point.

* Significance level when compared with the untreated control

** Significance level when compared with the samples with a single UVA dose

Figure 41

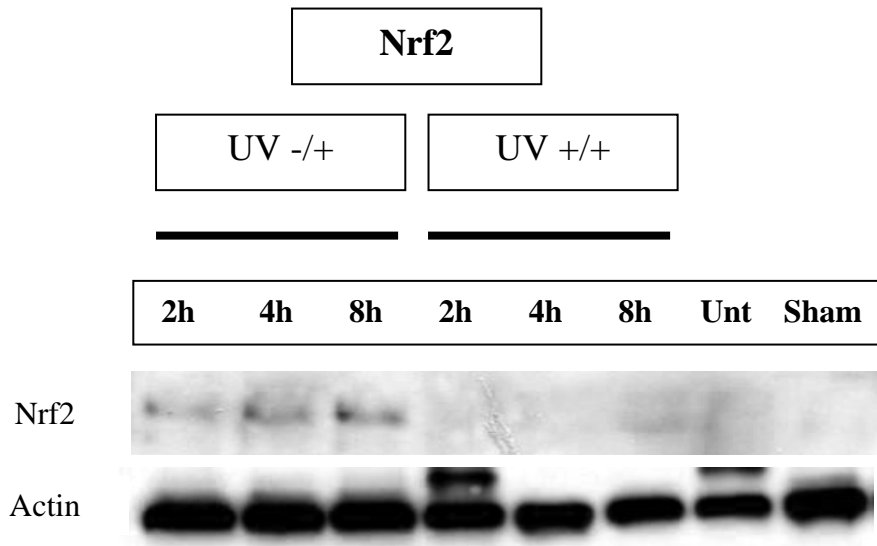


Figure 41 No Nrf2 protein induction following a second dose: FEK4 cells were UVA-preirradiated (250kJ/m^2) and incubated for 72 h at 37°C in conditioned media before a second irradiation under similar conditions. Nrf2 protein levels were quantified using Western blot analysis. Actin protein levels were determined in all samples and used for normalizing Nrf2 protein levels. Nrf2 levels in the samples treated with single UVA dose (UVA -/+) were compared with those in the doubly irradiated samples (UVA +/+), untreated sample (Unt) and Sham treated sample. The Sham sample was treated exactly the same as the UVA treated samples, but was not irradiated. This experiment was repeated 4 times with identical results.

3.10 Effects of SA and LMB treatments on UVA-mediated HO-1 induction

UVA treatment of FEK4 fibroblasts leads to an immediate release of heme from heme containing proteins (Kvam et al., 1999). Moreover, it has been shown in the murine model that heme not only decreases DNA binding capacity of Bach1 and removes Bach1 from the MARE sites but it also promotes stabilization of Nrf2 protein through prevention of ubiquitination (Alam et al., 2003). Interestingly it has been observed that depletion of heme prior to UVA irradiation by Succinyl Acetone(SA) treatment prevents UVA-mediated Nrf2 accumulation in FEK4 cells (Edwards and Tyrrell unpublished observation). In view of these observations we hypothesized that depletion of heme prior to UVA irradiation may prevent release of free heme, in the cellular environment following UVA irradiation. Therefore in the absence of free heme Nrf2 may not accumulate in the nucleus and Bach1 may not be released from the MARE site and this, in turn, may result in suppression of the HO1 gene. To test this hypothesis, FEK4 cells were treated with Succinyl Acetone(SA) in the serum free media for 18-22 h to inhibit heme synthesis. Following SA treatment, cells were treated with a physiologically relevant dose of UVA radiation (250kJ/m^2) and subsequently incubated in conditioned media for 12h. The level of HO-1 protein was monitored by Immunocytochemistry. As shown in figure 42, UVA treatment without SA pretreatment increased the level of HO1 protein compared to untreated cells. Significantly lower levels of HO-1 protein were observed following UVA treatment of cells that had been pre-treated with SA (figure 42 and 43).

These data are consistent with the idea that UVA-mediated release of free heme from the heme-containing proteins plays a central role in activation of the HO-1 gene and also support the concept that lack of heme release from the heme-containing proteins in the cellular environment following a second UVA dose may contribute in the development of refractoriness of the HO-1 gene to re-induction following a challenge UVA dose.

Crm1 is a nuclear export protein. It was hypothesised that inhibition of Crm1 protein may prevent UVA-mediated HO-1 induction. To test the hypothesis, cells were treated with an

inhibitor of Crm1, LeptomycineB (LMB) for 2 h prior to UVA irradiation. The cells were then UVA irradiated and incubated at 37°C for 12h. The level of HO-1 protein was checked by Immunocytochemistry following incubation. As described in figures 42 and 43, HO-1 protein levels in the cells without LMB pre-treatment were considerably higher following UVA irradiation compared to the cells with LMB pre-treatment. The data show that inhibition of the nuclear export pathway prevents UVA-mediated HO-1 activation.

Figure 42

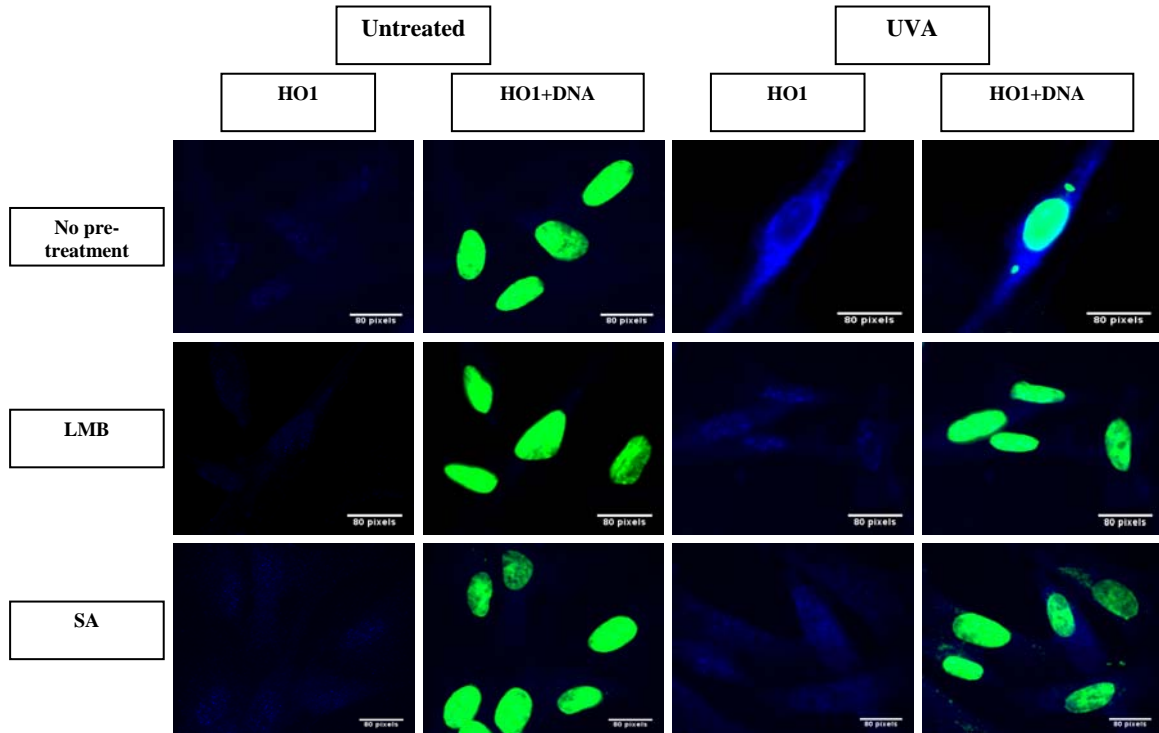


Figure 42 SA and LMB pre treatments prevent UVA-mediated HO-1 induction: FEK4 primary human fibroblasts given no pre-treatment, SA treatment and LMB treatment were UVA irradiated (250 kJ/m^2) and incubated in conditioned media at 37°C for 12h. The cells were fixed at the indicated time-points with 4% PFA and immuno-staining was performed using anti-Bach1 goat polyclonal primary antibody followed by Alexa Fluor 647 goat anti-mouse secondary antibody. The cell nuclei were stained with hoechst stain. The images for the HO-1 staining (Blue colour) and the nuclei (Green colour) were obtained with the 647nm filter and the UV filter settings respectively. Changes in the levels of HO-1 protein in cells with and without UVA treatment were compared with the cells with no pre-treatment, SA or LMB pre-treatments.

Figure 43

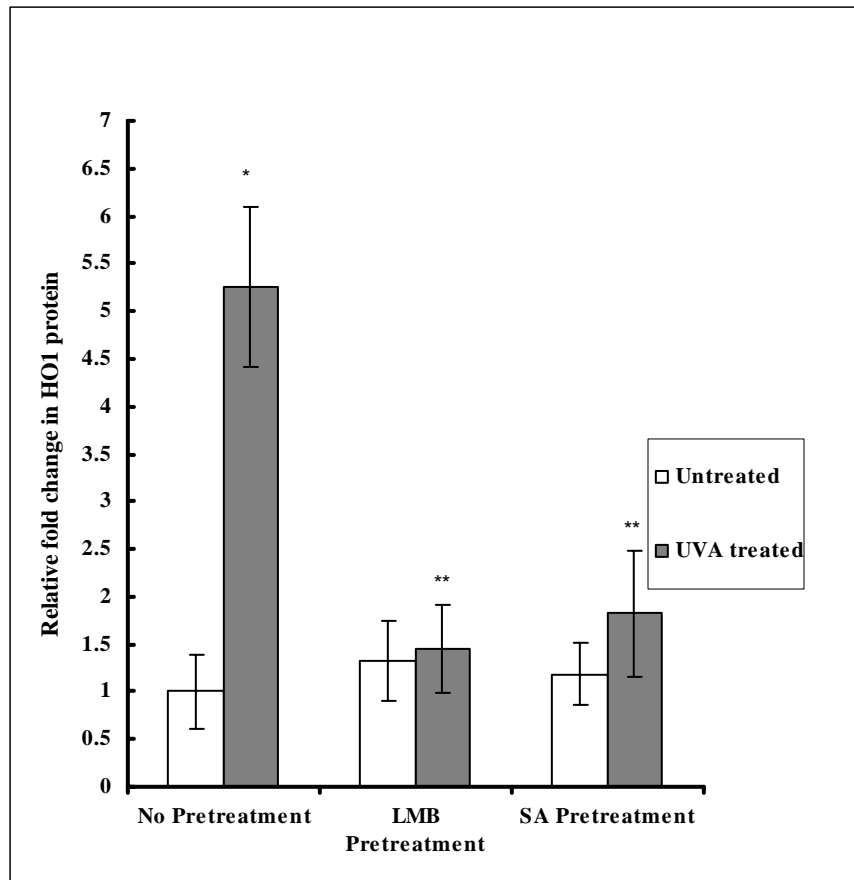


Figure 43 SA and LMB pre treatments prevent UVA-mediated HO-1 induction: Fluorescence intensity was quantified using the Image J software package. The mean gray value of 300 cells for each condition from three individual experiments was quantified for each sample and data were expressed as the relative fold increase over untreated samples. Data are mean \pm S.D. of 3 independent experiments. The Student paired t-test was used to evaluate the statistic significance ($p < 0.05$) of each data point.

* Significance level when compared with the untreated control

** Significance level when compared with the UVA irradiated samples with no pre treatment

3.11 The migration of HO-1 to the Nucleus

HO-1 has been characterised as a protein associated with the endoplasmic reticulum (ER). Both HO-1 and HO-2 have a hydrophobic region in their C-terminal regions which help them to anchor to cellular membranes (Shibahara *et al.*, 1985; McCoubrey, Jr. and Maines, 1993). Recently HO1 protein has been observed in other cellular compartments. HO1 protein signals in the cell nucleus have been observed following hypoxia, heme or heme/hemopexin treatments (Lin *et al.*, 2007). It has been shown that under these stress conditions, HO-1 cleaves and migrates to the cell nucleus. Further to this, it has been shown that the HO1 that has migrated in to the nucleus is enzymatically inactive. In FEK4 cells HO-1 protein expression is very low under normal condition and the protein is found in the cytoplasm. UVA irradiation increases HO1 expression several fold in the cell cytoplasm. However, when we treated cells with the Bach1 specific siRNA we observed a robust increase in HO-1 protein expression. Interestingly increased HO1 protein signals were observed in the nucleus (figure 44). HO-1 protein signals were also observed in the nucleus following UVA irradiation in the cells that had been given SA and LMB pre-treatments (figure 45 and 42). Taken together the data suggest that HO-1 may also localise in the cell nucleus. However, the functional role that nuclear HO-1 plays in UVA-mediated HO-1 regulation is not known.

Figure 44

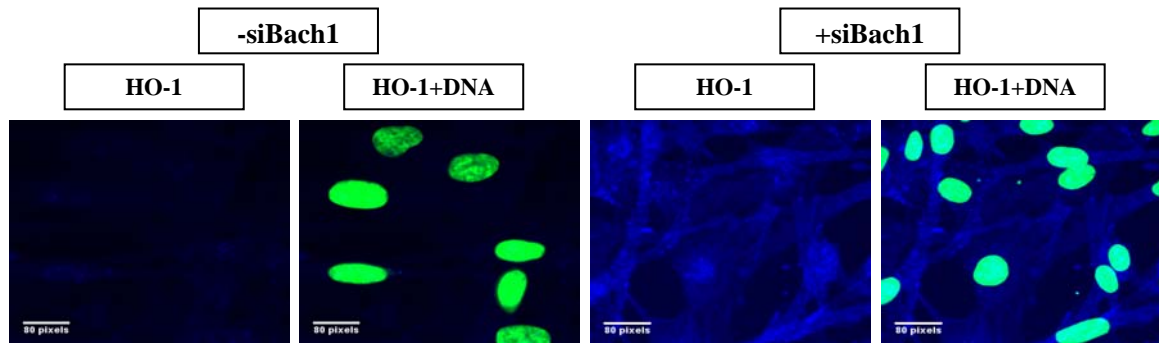


Figure 44 HO-1 protein signals in the nucleus: FEK4 cells were transfected for 4-6h with 5nM of Bach1 specific siRNA cocktail by using Lipofectamine 2000 reagent. Following transfection cells were further incubated for 48 h in conditioned media at 37°C. Following the incubation FEK4 cells were irradiated with a moderate dose of UVA (250kJ/m²) and incubated in conditioned media at 37°C for 12 h. The cells were fixed with 4% PFA and immuno-staining was performed using anti-HO-1 mouse monoclonal primary antibody followed by Alexa Fluor 647 goat anti-mouse secondary antibody. The images for the HO-1 staining (Blue colour) were obtained with the 647nm filter settings. Changes in the sub-cellular localisation of HO-1 proteins with and without siBach1 were compared before and after UVA irradiation.

Figure 45

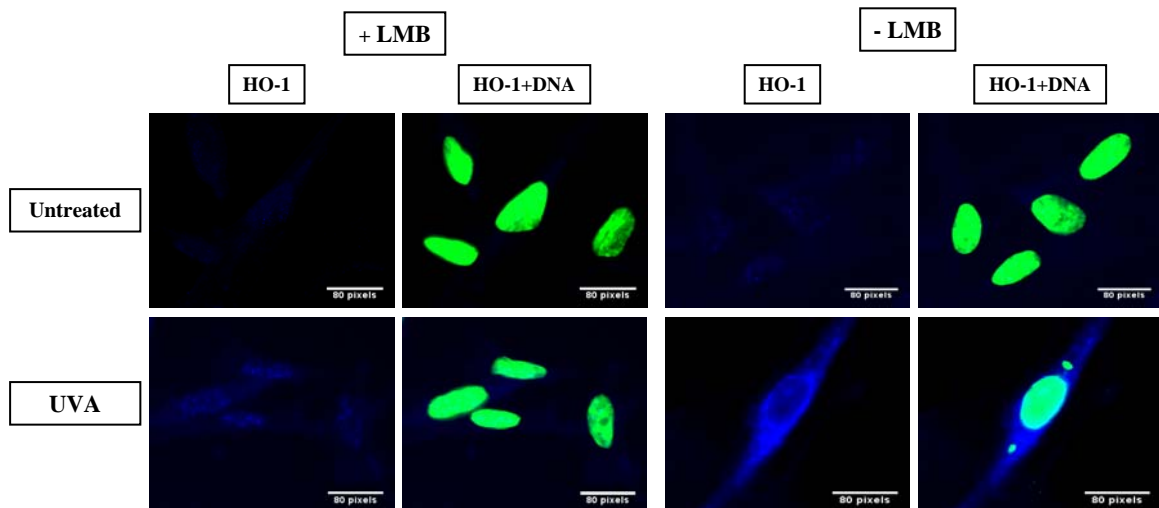


Figure 45 HO-1 protein signals in the nucleus: FEK4 primary human fibroblasts with no pre-treatment, SA treatment and LMB treatment were UVA irradiated (250 kJ/m²) and incubated in conditioned media at 37°C for 12h. The cells were fixed at indicated time-points with 4% PFA and immuno-staining was performed using anti-HO-1 primary antibody followed by Alexa Fluor 647 goat anti-mouse secondary antibody. The cell nuclei were stained with hoechst stain. The images for the HO-1 staining (Blue colour) and the nuclei (Green colour) were obtained with the 647nm filter and the UV filter settings respectively. Change in the sub-cellular localisation of HO-1 protein, with and without UVA treatment were compared with the cells with no pre-treatment or LMB pre-treatments.

4. Discussion

This study has examined the role of the transcription factor Bach1 in UVA mediated HO-1 gene regulation. We report that expression of the gene for the repressor protein, Bach1 is stimulated after moderate exposure to UVA irradiation. Endogenous Bach1 protein appears to be involved in UVA-mediated HO-1 expression and sub-cellular localisation of Bach1 plays a crucial role in UVA-mediated HO-1 expression. Heme released by UVA appears to play a central role in sub-cellular localisation of Bach1 and the heme status also contributes in the development of refractoriness (resistance of HO-1 to further induction by UVA).

Transcriptional regulation of the HO-1 gene involves NF-E2-Related Factor 2 (Nrf2) as a transcriptional activator and Bach1 as a negative regulator, both of which are involved in binding with small Maf proteins at MARE (Maf Recognition Element) sites in the promoter of the HO-1 gene. A simple model for the HO-1 gene activation is suggested whereby activation of the HO-1 gene occurs by derepression of the Bach1 repressor complex. Three separate mechanisms for derepression of Bach1 have been proposed. 1) Immediate nuclear export of Bach1 2) Heme-mediated degradation of Bach1 protein and 3) Heme-mediated inhibition of Bach1 DNA-binding. In this study it was observed that a moderate dose of UVA irradiation (250kJ/m^2) results in accumulation of Bach1 mRNA. It was further demonstrated that Bach1 protein accumulates in the nucleus following UVA irradiation indicating that UVA does not promote immediate nuclear export of Bach1. Furthermore, no decrease in Bach1 protein levels is observed and so it is unlikely that significant polyubiquitination and degradation of Bach1 occurs immediately after UVA irradiation. UVA irradiation promotes strong transcriptional activation of the HO-1 gene in human skin fibroblasts and UVA irradiation also leads to an immediate release of intracellular free heme from microsomal heme-containing proteins (Kvam *et al.*, 1999c). In view of these observations it is reasonable to postulate that UVA irradiation promotes derepression of Bach1-mediated repression. We propose that UVA-mediated release of free heme from heme-containing proteins may bind with the heme-binding protein Bach1 and titrate out Bach1 from the MARE sites and this reverses Bach1-mediated repression as an immediate response to UVA irradiation. Concentrations of heme as low as 0.03

μM (added exogenously) result in a slight inhibition of Bach1 DNA binding (Ogawa *et al.*, 2001b).

Bach1-a “stand-by” repressor

UVA exposure results in very strong HO-1 mRNA accumulation in human skin fibroblasts and this reaches a peak between 4 to 6 h post-irradiation incubation (Keyse and Tyrrell, 1989). Results in rodents cells show that UVA induces nuclear accumulation of Nrf2 protein immediately after UVA exposure (Hirota A, 2005). Results in primary human skin fibroblasts FEK4 also indicate immediate nuclear accumulation of Nrf2 protein following a similar dose (250kJ/m²) of UVA irradiation with a maximum level between 4-6 h (Edwards and Tyrrell, unpublished results). We reported here that UVA also induces Bach1 protein accumulation. Interestingly, we have noted that over-expression of Bach1 along with its binding partner represses basal and UVA-induced HO-1 protein expression. Since UVA induces the HO-1 gene while endogenous Bach1 still accumulates in the nucleus, Bach1 may be either inactive or the levels of Bach1 protein in the nucleus may not be sufficient to compete with Nrf2 under stress condition in order to bind with the small Maf proteins at the MARE sites. Therefore, it is likely that nuclear accumulation of *de novo* Bach1 protein may work as a “stand-by” repressor during UVA-mediated HO-1 activation. Induction of HO-1 degrades heme to CO, iron and biliverdin and reduces the cellular concentration of free-heme. Here we propose that in the cellular environment, where UVA-released free heme has been degraded by UVA-induced HO-1 protein, UVA-induced Bach1 probably plays a major role in the strong down regulation of the HO-1 gene. In support of this idea, it has been shown that exposure to TAR in combination with β-carotene cause an initial induction followed by a late repression of the HO-1 protein via the late nuclear accumulation of the Bach1 protein (Palozza *et al.*, 2006) and treatment of t-BHQ caused nuclear accumulation of both Nrf2 and Bach1, but the Bach1 accumulation was much delayed over that of Nrf2 (Dhakshinamoorthy *et al.*, 2005).

Since endogenous Bach1 protein remains dormant during UVA-mediated HO-1 induction, we further determined the role of Bach1 in UVA-mediated HO-1 expression by silencing Bach1 gene using siRNA specific to the Bach1 gene in

primary human skin fibroblasts. Silencing of Bach1 by Bach1 gene specific siRNA increases basal levels of the HO-1 protein and treatment with UVA radiation resulted in further increases in the level of HO-1 protein. This shows that under normal conditions, Bach1-mediated repression is dominant over the Nrf2-mediated activation and the repression complex maintains a constitutively low level of HO-1 protein expression. This finding is similar to that observed in Bach1 deficient mice (Sun *et al.*, 2002b). Interestingly, a previous report from our laboratory suggested that HO-1 is not inducible by UVA in primary human skin keratinocyte (Applegate *et al.*, 1995). Recent data from our laboratory indicates that basal levels of Bach1 are much higher in the human keratinocyte cell-line HaCat compared with primary human skin fibroblast and silencing of Bach1 by Bach1 gene specific siRNA robustly increases the HO-1 protein in HaCat cells indicating that in the keratinocyte lack of HO-1 induction is due to Bach1-mediated repression (Zhong and Tyrrell, unpublished data).

The transcriptional regulation of HO-1 not only depends on cell type and species but also the inducer. Hypoxia, desferrioxamine (DFO) and interleukin γ are other conditions and treatments which lead to activation of the Bach1 gene (Lee *et al.*, 1997b; Shibahara *et al.*, 2002a; Kitamuro *et al.*, 2003b). Elevated expression of Bach1 represses HO1 expression under these treatment conditions in HUVEC cells, human glioblastoma cells T98G and human lung cancer cells A549. Interleukin γ decreases HO1 expression via Bach1 induction in human retinal pigment epithelial cells, while hypoxia induces both HO1 and Bach1 mRNA expression. Hypoxia and interferon γ (INF γ) activates the mouse *ho-1* gene while these treatments repress the human *ho-1* gene (Lee *et al.*, 1997a; Shibahara *et al.*, 2002b; Kitamuro *et al.*, 2003a).

Role of HO-1 chromatin structure

Over and above the operation of transcriptional regulatory proteins, chromatin structures have a major influence on gene expression. It has been suggested that the acetylation status of histone H3 and H4 is an indication of the transcription status of genes; transcriptionally active regions are associated with hyperacetylated histones particularly methylation at K4 of histone H3, whereas transcriptionally silent regions are associated with hypoacetylated histones particularly methylation at K9 of histone H3 (Strahl and Allis, 2000; Bannister *et al.*, 2001; Lachner *et al.*, 2001; Santos-Rosa *et*

al., 2002; Nicolas *et al.*, 2003). In the case of the HO-1 gene, histone H3 is hyperacetylated at the enhancer regions of the HO-1 promoter under the normal conditions and the HO-1 gene is silenced by Bach1-MafK heterodimer. This means that the chromatin structure of HO-1 gene is in a preactivation state and this does not change further in response to an activation signal (Sun *et al.*, 2004a). By deploying this mechanism the HO-1 gene appears to bypass the histone modification stage. This allows a rapid on/off switching of the expression status of the gene in the event of stress conditions and/or post-stress conditions. In addition, the transcriptional activator, Nrf2, and the repressor, Bach1, both share a common binding partner MafK, therefore the HO-1 gene can undergo rapid transformation from an activated to a repressed state.

Role of heme in UVA-mediated HO-1 regulation

Heme oxygenase 1 plays a crucial role in the breakdown of heme to bilirubin, carbon monoxide and iron. Heme is a prosthetic group of various proteins which are involved in various important biological processes. In addition heme is now a well-characterised signalling molecule in gene regulation (Furuyama *et al.*, 2007). Interestingly, heme plays a central role in regulation of proteins involved in heme synthesis and heme degradation. For example higher concentration of heme inhibits heme synthesis by repressing expression of ALAS1, the first enzyme in the heme synthesis pathway (Anderson *et al.*, 2001).

With respect to heme catabolism, heme plays a central role in regulation of UVA-mediated HO-1 expression. Cellular concentrations of free heme determine expression levels of HO-1 by regulating both the activator factor (Nrf2) and the repressor factor (Bach1). In the case of UVA irradiation a previous report from our laboratory has demonstrated that UVA releases heme from heme-containing proteins (Kvam *et al.*, 1999b). Heme regulates Bach1 by inhibiting its DNA-binding capacity (Ogawa *et al.*, 2001a) and promoting nuclear export of the protein (Suzuki *et al.*, 2004b).

In this study we show that Bach1 protein accumulates in the nucleus following UVA irradiation indicating that the immediate release of heme by UVA may inhibit DNA-binding activity of Bach1 but UVA does not seem to provoke rapid export from the

nucleus. In support of this observation, detailed analysis of heme-Bach1 binding by heme titration revealed that CP5-6 influences Bach1 DNA-binding. Furthermore, the bZIP domain of the Bach1 protein which is responsible for DNA binding is situated in between CP5 and CP6. Therefore it is proposed that heme binding at CP5 and CP6 may undergo conformational change and reduce DNA-binding capacity of Bach1 by shielding the bZIP domain of Bach1 (Hira *et al.*, 2007c). In view of these observations, it is likely that UVA-mediated immediate release of heme appears to bind with CP5 and CP6 and releases Bach1 from MARE sites.

It appears that UVA-mediated heme release provoked much delayed Bach1 nuclear export following UVA irradiation since Bach1 nuclear export only occurs 12h following UVA irradiation. We have further demonstrated that heme-depletion by SA or LMB treatments prior to UVA irradiation prevents the nuclear export of Bach1 indicating that heme is responsible for Crm1-mediated Bach1 nuclear export. Interestingly it has been shown that CP3, CP4 and a hydrophobic region between CP3 and CP4 are particularly involved in heme-mediated Bach1 export (Suzuki *et al.*, 2004). It is proposed that under normal conditions the hydrophobic region which has sequence homology with conventional nuclear export signal (NES), is masked by CP3 and CP4 in order to prevent the nuclear export. Under stress conditions two molecules of heme bind at CP3 and CP4 in order to induce heme dependant nuclear export via conformational changes in the Bach1 protein (Hira *et al.*, 2007b). In addition, at higher heme concentrations, heme-Bach1 complex exhibited an absorption spectrum with a major Soret peak at 371 nm and Raman band at 343 cm^{-1} compared to a spectrum containing the major Soret peak at 423 nm at lower levels of heme (Hira *et al.*, 2007a). In view of these observations, we propose that UVA-mediated inhibition of DNA-binding capacity of Bach1 and UVA-induced nuclear export occur at different cellular concentrations of heme and may involve two distinct types of heme-Bach1 binding.

In addition, a previous study from our laboratory has demonstrated that UVA irradiation promotes UVA-mediated nuclear accumulation of the Nrf2 protein (Edwards and Tyrrell, unpublished observation). Heme plays a central role in regulation of Nrf2 by increasing the stability of the protein (Sun *et al.*, 2004b). Results from this laboratory have shown that SA treatment prevents UVA-mediated

accumulation of Nrf2 protein and decreases stability of the Nrf2 protein (Edwards and Tyrrell, unpublished results). We now show that depletion of heme by SA treatment prior to UVA irradiation results in a significant decrease in UVA-mediated HO-1 induction. These data are consistent with the idea that elevated levels of free heme (a pro-oxidant) in the cellular environment following UVA irradiation not only induce derepression of Bach1-mediated repression and Bach1 nuclear export but also promote nuclear accumulation and stabilisation of Nrf2. In view of these observations, we propose that UVA-mediated release of heme from heme-containing proteins plays a central role in UVA-mediated HO-1 regulation.

Heme-independent nuclear export of Bach1

Bach1 is also exported from the nucleus by a heme-independent mechanism. If cells are treated with cadmium or arsenite, Bach1 is immediately exported from the nucleus. In the case of cadmium-mediated nuclear export, several mechanisms appear to be involved (Suzuki *et al.*, 2003a). The cytoplasmic localisation of Bach1 following cadmium treatment is mediated via the BTB region, the CLS region and the 636-685 amino acid region of the protein. Cadmium-mediated activation of the ERK1/2 of MAPK pathway may also contribute to Bach1 cytoplasmic localisation and activity (Suzuki *et al.*, 2003b). The involvement of the BTB and CLS regions of Bach1 in UVA-mediated Bach1 nuclear export is not known. Small Maf proteins may play an active role in Bach1 export. Observations in the current study show that overexpression of Bach1-GFP vector alone in FEK4 cells results in Bach1 protein expression in cytoplasm with clear nuclear exclusion of Bach1 whereas co-transfection of Bach1-GFP along with MafK-RFP resulted in Bach1 protein accumulation in the nucleus indicating that MafK is essential for nuclear accumulation of Bach1. Similar observation has been reported in the rodent cells (Suzuki *et al.*, 2003c). Therefore lack of small Maf protein 12h following UVA irradiation may play a role in UVA-mediated Bach1 export.

A model for UVA-mediated human HO-1 regulation in human skin fibroblast is summarised in Figure 46-49. Under normal unstressed conditions where the intracellular “free” heme level is very low, the Bach1 mediated repressor complex occupies the MARE sites (figure 46). The enhanced basal level of HO1 that results from Bach1 silencing indicates that under normal conditions, Bach1 mediated repression is dominant over the Nrf2 mediated activation in human skin fibroblasts and the repression complex maintains a constitutively low level of HO1 protein expression. This is similar to observations in Bach1 deficient mice(Sun *et al.*, 2002a) and in human liver cells(Ghaziani *et al.*, 2006). Under normal conditions cytoplasmic protein Keap1 suppresses Nrf2 activity by proteolytic degradation and cytoplasmic retention (figure 46).

Figure 46

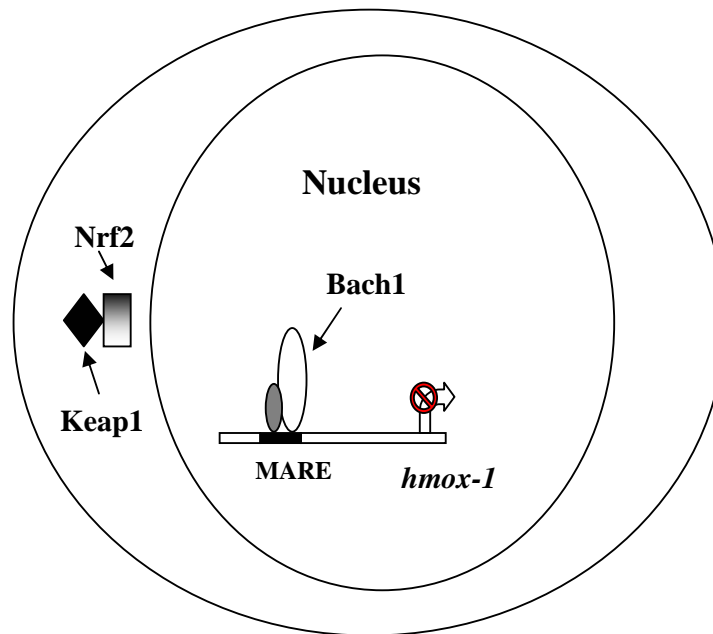


Figure 46 In normal (homeostatic) conditions, Bach1 protein stays in the cell nucleus and occupies the MARE sites of the HO1 promoter in order to keep the HO1 gene suppressed, while Nrf2 activity is inhibited by Keap1 binding in the cytoplasm.

When cells are exposed to UVA irradiation (Figure 47), UVA releases free cellular heme from heme-containing proteins and causes an increase in the free cellular heme concentration. It has been shown that heme plays a central role in regulation of the dynamic exchange of both Bach1 and Nrf2 (Sun *et al.*, 2004c). This increased level of intracellular heme appears to release Bach1 from MARE sites by inhibiting DNA-binding capacity of Bach1. UVA appears to release Nrf2 from Nrf2-Keap1 complex which accumulates in the nucleus. Further to this, nuclear accumulation of Nrf2 is essential for UVA-mediated HO-1 induction. UVA-mediated heme release increases stability of Nrf2 which binds to Maf and occupies the MARE sites to initiate activation of the HO-1 gene. Interestingly, when heme synthesis was inhibited by SA treatment, a lack of Nrf2 accumulation in the nucleus was observed (Edwards and Tyrrell, unpublished observation) and this appears to decrease UVA-mediated HO-1 activation. UVA treatment also activates transcription of the Bach1 gene and leads to accumulation of the protein in the nucleus which appears to work as a “stand-by” repressor. Unlike heme, cadmium or arsenite treatments, UVA did not cause immediate nuclear export of Bach1 or a decrease in the level of the protein indicating that the increased intracellular heme level does not affect de novo synthesis of Bach1 or its degradation immediately after UVA treatment.

Figure 47

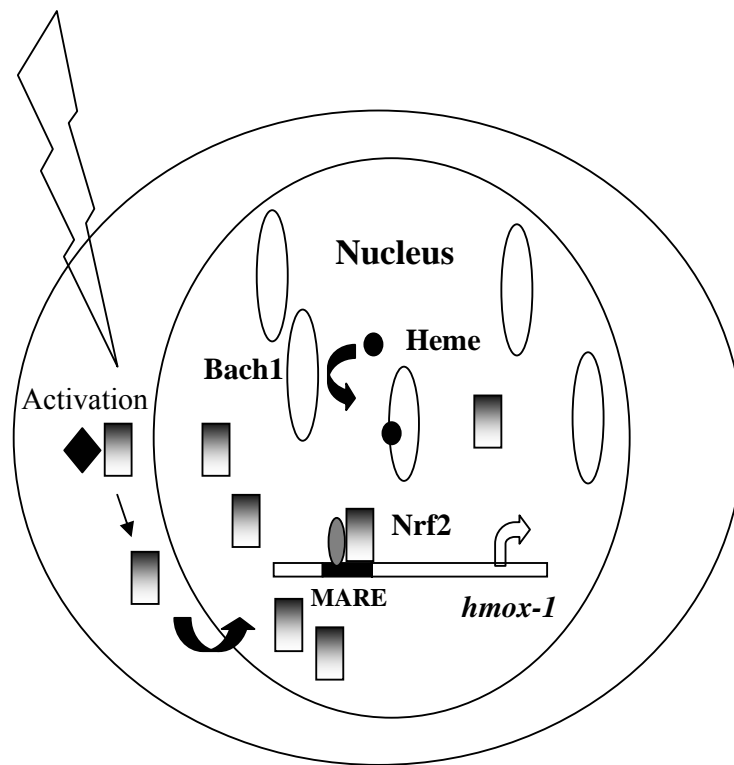


Figure 47 When cells are exposed to UVA irradiation, UVA releases heme (black dot) from the heme-containing proteins. This heme may bind to the MARE-bound Bach1 protein leading to release of its binding from MARE sites. UVA irradiation also promotes a strong and rapid accumulation of Nrf2 protein in the nucleus which occupies the MARE site to activate the HO-1 gene. Simultaneously, UVA irradiation induces Bach1 gene expression and promotes de novo synthesis of the Bach1 protein which also accumulates in the nucleus.

Following UVA treatment Bach1 is exported from the nucleus only after 12h post-irradiation incubation (figure 48). Nuclear export of the endogenous Bach1 protein is prevented in UVA-treated cells that have been depleted of heme (by SA treatment) leading to the conclusion that the export of Bach1 following UVA irradiation is mediated by heme. The BTB and CLS domains may also influence UVA-mediated Bach1 nuclear export but the underlying mechanism is not known.

Figure 48

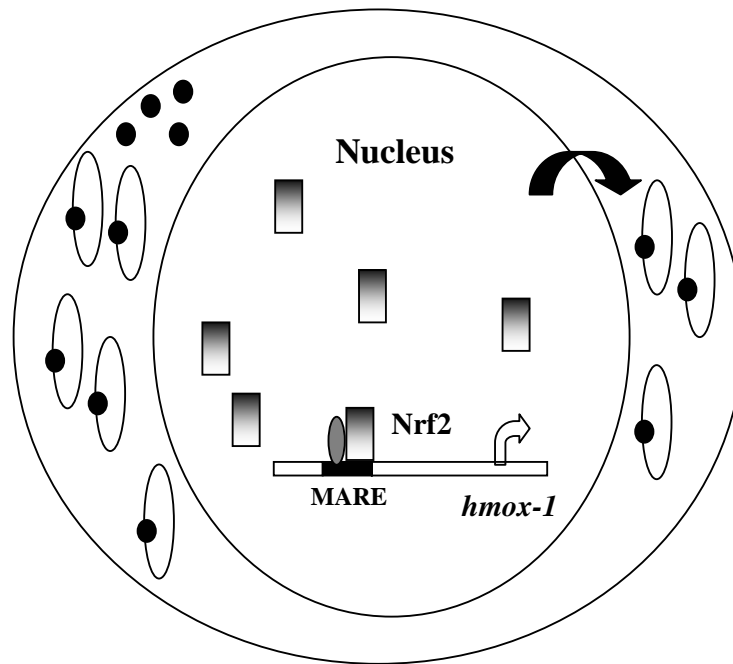


Figure 48 UVA released heme reaches a critical concentration between 10-12 h following UVA irradiation and this promotes nuclear export of Bach1 protein.

A model for refractoriness to UVA re-induction of HO-1

Heme was postulated to be a crucial factor in the development of refractoriness of the HO-1 gene to the re-induction by a challenge dose of UVA(Noel and Tyrrell, 1997). The loss of total hemoprotein via UVA mediated degradation or as a result of degradation by increased levels of HO-1 following UVA treatment do not recover within 48 h (Kvam *et al.*, 1999a). Interestingly, one recent report suggested that CO may also play a central role in heme release. It has been demonstrated that CO, a by-product of the HO-1 enzymatic reaction, can bind to heme-containing protein, haemoglobin and the binding of CO prevents oxidation of haemoglobin and prevents the release of free heme from haemoglobin(Pamplona *et al.*, 2007). Complementing the model of refractoriness where heme plays a central role, we have now shown a lack of Nrf2 induction and its nuclear accumulation following the challenge dose of UVA (figure 49). It has been reported that following activation of Nrf2 target genes, the cytoplasmic protein Keap1 promotes Nrf2 degradation (Nguyen *et al.*, 2005). In addition, Nrf2 activity is regulated by heme which increases the stability of the short lived protein (Alam *et al.*, 2003). We observed a very low level of Nrf2 protein 48 h following UVA irradiation so that Nrf2 protein appears not to recover within two days of treatment (figure 49). It is likely that both Keap1 mediated proteolysis and the lack of heme protein as a source of heme to be released following the challenge dose, contribute to the lack of Nrf2 induction and nuclear accumulation.

In contrast to Nrf2, we observed higher levels of Bach1 protein in the nucleus 48 h after UVA treatment indicating that Bach1 protein which had been exported in between 12h and 24 h following the treatment, returns to the nucleus in the absence of free heme and probably plays a major role in the strong down regulation of the HO1 gene by occupying the vacant MARE sites (figure 49). Therefore it is reasonable to propose that, in the absence of free cellular heme, Bach1 may not be removed following the challenge dose. The challenge dose further increases Bach1 protein accumulation in the nucleus. The absence of nuclear Nrf2 and the lack of free heme in the cellular environment as well as the *de novo* synthesis of Bach1 and its nuclear accumulation together appear to be responsible for the development of refractoriness to re-induction of HO-1.

Figure 49

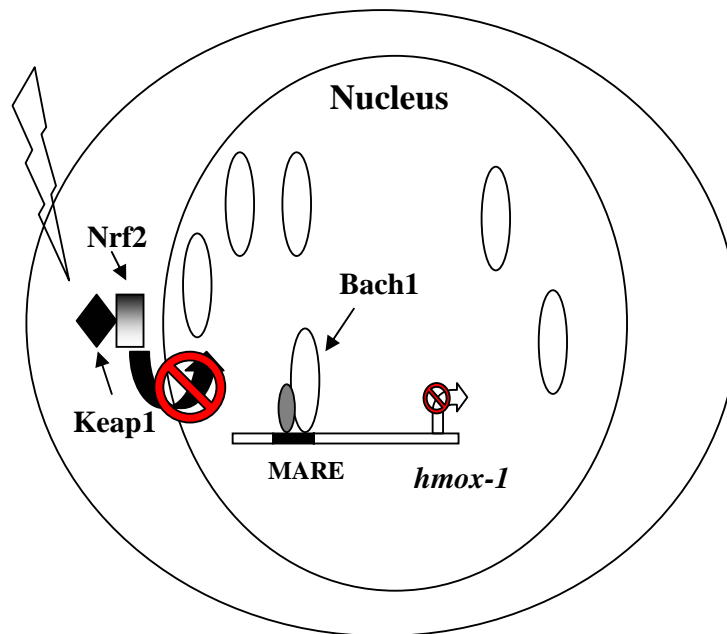


Figure 49 48 h following UVA irradiation, Nrf2 protein returns to the cytoplasm and Bach1 protein returns to the nucleus and may now occupy the MARE sites in the absence of Nrf2 protein in the nucleus. Following a challenge dose, Bach1 further accumulates but no induction or accumulation of Nrf2 protein was observed in the nucleus.

In summary, this study has highlighted the role of the repressor protein Bach1 in UVA mediated HO-1 regulation in primary human skin fibroblasts. Our results are consistent with the concept that UVA radiation activates Bach1 gene expression and causes *de novo* synthesis of Bach1 protein which accumulates in the nucleus. Our findings suggest that Bach1 is not only responsible for maintaining the constitutively low levels of HO1 but also appears to have a role both in the strong down-regulation of HO1 once the HO1 protein has been activated and in the phenomena of refractoriness to re-induction. So far HO-1, NQO1, GSH, both alpha and beta globin genes as well as the iron storage protein ferritin are known targets of Bach1 and have very important roles in various important biological properties. Since HO-1 has cytoprotective, anti-oxidant, anti-apoptotic and anti-inflammatory properties, Bach1 appears to be a potential target for therapies based on HO-1.

5. References

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